ROLES OF DNA METHYLTRANSFERASE 4 DURING ZEBRAFISH DEVELOPMENT

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

The research work presented in this dissertation describes tissue-specific functions of *DNA methyltransferase 4* (an ortholog of the mammalian DNTM3B) during zebrafish hematopoiesis. Studies have implicated the involvement of DNA methylation in regulating hematopoiesis. Although DNA methylation by Dnmt1, Dnmt3a and Dnmt3b is required for HSC maintenance and self-renewal, the exact contributions of these enzymes in commitment of HSCs to different blood lineage precursors and terminal differentiation of these precursors into mature cells are not known.

Answers to this idea are important as deregulations of DNMT1, DNMT3A and 3B are known to be involved in hematopoietic diseases. Thus, a better understanding of the contributions of DNMTs in regulating normal hematopoiesis will help in designing better drugs for treating hematopoietic diseases that arise due to aberrant methylation patterns.

Chapter 1 is an introduction to DNA methylation and the enzymes, DNA methyltransferases that confer this methylation mark. This chapter summarizes literature from various model systems that allude to the requirement of DNA methyltransferases in a tissue-specific manner during normal development.

Chapter 2 describes the utility of zebrafish as a model system to study DNA methylation. Also, I have characterized the embryonic expression patterns,

morpholino knockdowns of DNA methyltransferases 4, 5, 6, 7, and 8 during zebrafish development.

Chapter 3 discusses the epigenetic regulation of mature blood lineages by DNA methyltransferase *dnmt4* in zebrafish. Here we show that *dnmt4* is expressed in the hematopoietic compartment of zebrafish embryos. Transient knockdown of *dnmt4* confers terminal differentiation defects of the erythroid and the myeloid cells. The data discussed in this chapter juxtapose *dnmt4* morphants as an amenable model system to study the molecular pathways affected in (***I***mmunodeficiency ***C***entromere instability and ***F***acial anomalies) ICF type-1 syndrome, which arises due to missense mutations in human DNMT3B.

Chapter 4 describes the epistatic relationship of *dnmt4* with the tumor suppressor gene *apc* in regulating zebrafish hematopoiesis.

Chapter 5 presents the main conclusions of my dissertation work. I have described the implications of my findings and the future directions of this work.

To my loving father Syed Abid Ali Jafri, may you rest in peace.

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NOMENCLATURE

GENE	human gene
Gene	mouse gene
gene	zebrafish gene
GENE	human gene product
GENE	mouse gene product
Gene	zebrafish gene product

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CHAPTER 1

DNA METHYLATION IN DEVELOPMENT AND DISEASE

1.1 DNA methylation

Generally in a multicellular organism with the exception of T and B lymphocytes that lose DNA sequences during VDJ recombination by deletions (1) most cells have exactly the same DNA sequences. In spite of this, the organism has various cell types that differ both in structure and in function. This cell specificity in part can be attributed to tissue-specific transcription factors and epigenetic changes, which are heritable modifications that occur outside the DNA sequence. Epigenetic mechanisms that regulate the genome include DNA (2-4) and histone (5) modifications, inclusion of histone variants (6) and noncoding RNAs (7,8).

Of these modifications DNA methylation plays a number of important functions in regulating the mammalian genome. Some of the biological functions that DNA methylation is implicated in are gene expression (9-11), cellular differentiation and development (12-14), genomic imprinting (15,16), X chromosome inactivation (17,18), silencing of retrotransposons and tandem repetitive sequences (19), preventing genomic instability (20), development of

autoimmune rheumatic diseases (21), cellular reprogramming (22), behavior (23), brain development (24), immune functions (25) and aging (26).

Importantly deregulations of the methylation machinery and aberrant DNA methylation patterns are associated with imprinting defects (15, 27), and various diseases like (***I***mmunodeficiency ***C***entromere instability and ***F***acial anomalies) ICF syndrome (28) and various cancers (29). Unlike mutations or changes that involve alterations in DNA sequence, DNA methylation is a reversible process. This makes DNA methylation an important target for drug development.

1.2 DNA methylation in the context of CpG dinucleotide

DNA methylation can occur in CG, CHG and CHH context (where H can be C, T or A). In mammals DNA methylation is usually referred to as the covalent addition of a methyl group at the fifth position on the cytosine ring primarily in a palindromic 5'-CpG-3' dinucleotide context (30) and occasionally in non-CpG dinucleotide context (31-33) (Figure 1.1).

The human genome has 56 million CG dinucleotides of which 60 to 80% are methylated (34). However, the CpG dinucleotides are underrepresented in the genomes of mammals by 5- to 10-fold in comparison to the other dinucleotides because of deamination of 5mC to T thus creating TG mismatches which can be fixed as C to T transitions (35). Interestingly, unmethylated cytosines deaminate nearly four times slower than 5mC to uracil, creating UG mismatches which are efficiently removed from the DNA by uracil glycosylases

(36). As a result of this preferential deamination the human genome is CG dinucleotide deficient.

Also, the occurrence of CpG dinucleotides is non-random. While most regions of the mammalian genome are CpG poor, repetitive DNA sequences and regions called CpG islands harbor CpG dinucleotides nearly at their statistically expected levels (37).

By definition a CpG island is a stretch of DNA that is on average 1000bp long, has a G+C content of at least 50% and has a ratio of observed CpG/expected CpG more than 60%. In the non-CpG island regions of the genome this ratio is as low as 0.1 to 0.2 (37). In the human genome 50% of all genes harbor CpG dinucleotides in their promoters (38) (Figure 1.2).

However, for most ubiquitously active genes the CpG dinucleotides in the transcription start site (TSS) associated with promoters are constitutively unmethylated and gene bodies are heavily methylated (34,39,40). The methylation of the gene body occurs perhaps to prevent spurious transcription initiation leading to formation of truncated proteins. Also the unmethylated CpG dinucleotides may be a defense mechanism to protect the promoters of genes from spontaneous mutation because of to 5mC to T transitions.

It has been observed that CpG dinucleotides are sometimes methylated in a tissue-specific manner but these methylation profiles are not inherited from the germ cells (34,41-45). DNA methylation of the promoter often correlates with gene repression in a tissue specific manner (34,39,42). As a rule of thumb, the CpG islands associated with imprinted genes and genes associated with X

chromosome inactivation, and inactivated tumor suppressor genes in pathological conditions are almost always silenced by hypermethylation.

1.3 DNA methylation in development

Mammalian genomes witness genome-wide epigenetic reprogramming *via* waves of DNA methylation and DNA demethylation at two distinct stages. In mice it is first observed in the primordial germ cells (PCGs) around E10.5 to E13.5 and then from the newly fertilized egg to the morula stage before preimplantation (46). At the time of primordial germ cell maturation into sperm and eggs the highly methylated PGCs undergo genomewide demethylation in both male and female germ cells, which completes by E13 and E14 (47,48). Remethylation in the male PCG occurs at prospermatogonia stage (E15 to E16), in females the remethylation occurs in the growing oocyte (47,48). Perhaps this wave of demethylation is required to erase and establish imprints according to the sex of the germ line.

The genes responsible for active DNA demethylation have been a controversial issue. Recently in zebrafish embryos a complex of *aid*, *mbd4* and the *gadd45* proteins was shown to be involved in active DNA demethylation of exogenous, methylated plasmids (49). This study confirmed the existence of a DNA demethylation complex. Also, it is likely that this and other complexes may be involved in epigenetic reprogramming of the methylome during embryonic development. Interestingly, a novel class of proteins, the ten-eleven translocation (TET), belonging to the class of enzymes methylcytosine dioxygenase have been

shown to be involved in epigenetic reprogramming *via* regulating the 5-hydroxymethylcytidine (5hmC) content of the genome (50).

At fertilization the bulk methylation levels of the sperm are higher than that of the egg. The sperm genome is demethylated shortly after fertilization (48), and the genome of the newly developing zygote experiences a wave of reprogramming. After this second wave of demethylation in mammalian development *de novo* methylation occurs in selective intermediate cell mass (ICM) cells at the time of implantation to establish embryonic lineages and cell identities (51). At this time the bulk methylation of the developing zygote is equivalent to the bulk methylation levels of the sperm. However, how these new methylation marks are established in tissue specific genes is not clear. Figure 1.3 summarizes the dynamics of 5mC content during embryonic development.

It is postulated that the second wave of demethylation helps in erasing the paternal and maternal methylation signature to set up new methylation marks in the genome of the developing embryo that will be distinct from that of the parents. In mammals this early wave of embryonic demethylation is mostly conserved. However, there are some variations in timing or extent of demethylation. Interestingly, it is still not clear if there is a genome-wide paradigm for this re-establishment phase although some tissue-specific genes (44,52), and pluripotency genes (53) are targets of promoter methylation. Importantly, the factors that dictate the new differential methylation patterns, and the recruiters of the different DNA methyltransferases to these loci are not clearly identified.

1.4 DNA methylation in a non-CpG dinucleotide context

One of the non-CpG methylation marks that has recently been discovered is 5-hydroxy methylcytosine or 5hmC within the mammalian neuronal and embryonic stem cell genomes (50, 54). This modified base is also called the sixth nucleotide and is formed by the conversion of 5mC by the protein ten-eleven translocation 1 (TET1), belonging to the class of enzymes methylcytosine dioxygenase (50). Figure1.1 shows the structure of the 5-hydroxymethylcytidine.

TET1 utilizes 2-oxoglutarate (2OG)- and Fe (II) to oxidize 5mC to 5hmC (50). In ES cells TET1 protein associates and colocalises with SIN3A co-repressor complex (55). However, of 5hmC is not only present at promoters and CpG islands (3) but also at enhancers and gene bodies (56). Because 5hmC is associated with euchromatic DNA it is thought to be an activating mark (3). Interestingly, pre-existing 5mC marks are required for the formation of the 5hmC residues (3). This again highlights the importance of 5mC in the mammalian genome. Perhaps the occurrence and the genomic distribution of 5mC lay down the blueprint for different modifications during this epigenetic reprogramming.

In genome wide studies in ES cells 5hmC has been shown to enrich at OCT4 and NANOG binding sites (56-58), promoters of genes that harbor both repressive H3K27me3 and the activating H3K4me3 histone marks (59) and at enhancers bearing activating H3K4me1 and H3K27ac marks (56). Also, it has been suggested that 5hmC levels are dynamic during development, its levels first increase in murine ES cells and then decrease on differentiation into embryoid bodies (4). 5hmC is expressed in the brain and heart of human and murine

tissues, suggesting that it may be required in a tissue-specific manner to regulate gene expression (4). Other than these two modifications ES cells also harbor some cytosine methylation at CpA and CpT sites (31) although the functions of these modifications are not clear.

The above data suggest that non-CpG methylation may also play a number of functions in regulating the chromatin architecture during development and disease.

1.5 DNA methylation and cell differentiation

All cells of a multicellular organism are similar at the genetic level. However, the cells have distinct structures and functions depending upon the type of genes they express. The regulation of gene expression in different cell types and different tissue types of a multicellular organism is controlled in part by DNA methylation.

However, one school of thought argues that methylation or de-methylation of promoters of genes cannot be the primary effectors of cellular differentiation. According to this theory cellular differentiation occurs in both kinds of organisms, those that have DNA methylation (vertebrates, fungi and flowering plants) and those that show very little evidence of DNA methylation (*Caenorhabditis elegans Sachharomyces cerevisea* and *Drosophila melanogaster*).

Thus it has been hypothesized that, unlike DNA methylation that is a non-conserved cellular mechanism, differentiation is more likely to be controlled by biological processes that are conserved through evolution. However, mounting

evidence points in favor of DNA methylation regulating differentiation. Studies indicate that genes have differential methylation in different tissue types during embryonic development, suggesting that DNA methylation indeed may be required during differentiation (60-63).

1.6 Mammalian DNA methyltransferases

Mammals harbor three catalytically active DNA methyltransferase proteins called DNMT1, DNMT3A and DNMT3B. The third member of the DNMT3 family is DNMT3L that is devoid of DNA methyltransferase catalytic activity. The generic DNA methyltransferase protein consists of two main parts, a multidomain N-terminal regulatory region that varies in size depending upon the DNMT and a highly conserved C-terminal catalytic domain. Figure 1.4 shows the different domains of the murine DNA methyltransferases.

The highly divergent N-terminus of the protein mediates interactions with DNA, chromatin and other proteins. This region of the protein also helps in nuclear localization. The C-terminal part of the DNMTs is smaller but highly conserved across species. It has the 10 amino acid motif that serves as a signature for all DNA methyltransferases (79). The catalytic domains have the AdoMet-dependent MTase fold and this region helps both in cofactor binding and catalysis. The transcription repression domain (TRD) is non conserved among the DNMTs and it helps in DNA recognition and specificity (80).

1.6.1 Enzyme classification

Based on their structure the DNA methyltransferases are divided into two main classes: the DNMT1 and DNMT3 family. Based on their function the DNA methyltransferases can be categorized into two main classes, the maintenance and the *de novo* methyltransferases. The maintenance class prefers hemimethylated DNA as substrate and helps in maintaining the methylation status in dividing cells in every replication cycle. DNMT1 in mammals is deemed the maintenance methyltransferase. It has 10- to 50-fold higher preference for hemimethylated DNA over unmethylated DNA as a substrate *in vitro* (81, 82).

De novo methyltransferases are the ones that methylate CpG dinucleotides at completely unmethylated substrates. DNMT3A and DNMT3B are the *de novo* DNMTs. It has been shown that DNMT3A and DNMT3B are the enzymes that hypermethylate and silence viral repeat sequences as well (83, 84).

However, the distinctions based on substrate preference are not absolute. DNMT1's specific activity on unmethylated substrates is much greater than that of DNMT3A and DNMT3B. Importantly, overexpression of Dnmt1 in mice leads to aberrantly methylated regions of imprinted loci suggesting that Dnmt1 in this scenario can act as a *de novo* DNA methyltransferase. This distinction also fails in the case of *DNMT1* deficient cell lines where in the absence of *DNMT1* methylation levels in the genome are maintained. This suggests that other methyltransferases may also act as maintenance methyltransferase (85, 86).

1.6.2 DNMT1

Dnmt1 was the first mammalian DNA methyltransferase gene cloned (87) and characterized biochemically. The preference of *Dnmt1* for hemimethylated DNA can be attributed to its localization at the replication fork during the S phase (88, 89). Figure 1.4 shows the domain structure of *Dnmt1*. DNMT1 is a large protein of 1620 amino acids in mouse and 1616 amino acids in humans. The N- and C-termini are connected through lysine-glycine repeats. The N-terminal regulatory region has various domains that that may help in mediating its functions.

The main N-terminal regulatory domains of DNMT1 are 1) DMAP1- (DNA methyltransferase associated protein 1) interaction domain; 2) PBD domain- PCNA (proliferating cell nuclear antigen) binding domain; 3) Three independent NLS sequences; 4) TS targeting sequence domain; 5) CXXC containing zinc-finger-binding domain between the PCNA-binding and the BAH regions. Dnmt1 has eight cysteine residues in two CXXCXXCXXC motifs. 6) BAH 1 and 2 domains- (Bromo-adjacent homology) and 7) KG (series of lysine and glycine residues) linker localized between N- and C- terminal part of the enzyme.

The C-terminal catalytic domain of *Dnmt1* requires the other motifs to perform its function both *in vitro* and *in vivo* (90). The expression of mammalian DNMT1 protein is affected by a number of factors both in disease and development. Some of these regulatory controls are mediated by miR-148 (91-93), miR-152 (91) and miR-126 (94). In addition posttranslational modifications

like phosphorylation, methylation, acetylation, ubiquitination, and sumoylation affect the stability, activity and turnover of the DNMT1 protein (95).

1.6.3 DNMT3

The mammalian DNMT3 family has three main members: DNMT3A, DNMT3B and DNMT3L. DNMT3A and DNMT3B establish the methylation patterns required for early development. These enzymes are also required to establish methylation patterns in the primordial germ cells. DNMT3L is catalytically inactive and functions as a cofactor for DNMT3A and DNMT3B. Its function is required in germ cells.

Dnmt3a and *Dnmt3b* are *de novo* DNA methyltransferases, however *in vitro* they do not show significant preference for hemimethylated or unmethylated DNA as substrate (84, 96). Interestingly, although DNMT3 family members are primarily considered as *de novo* DNA methyltransferases, they have been shown to play a role in maintaining methylation at heterochromatic DNA sequences (97).

Although there may be some functional over lap in the functions of *Dnmt1*, *Dnmt3a* and *Dnmt3b* they have distinct tissue-specific functions, too. ICF syndrome is caused by mutations in the *DNMT3B* gene and *DNMT1* or *DNMT3A* do not complement for the reduced activity of DNMT3B. The fact that hypomorphic DNMT3B is sufficient to cause this disease suggests that DNA methyltransferases may have discrete functions during development.

Like *Dnmt1*, the *Dnmt3* family possesses a conserved C-terminal catalytic domain containing C5 DNA methyltransferase motifs. The N-terminal regulatory

domain of *Dnmt3a* and *Dnmt3b* are unrelated. The N-terminal has the 1) Cysteine rich ADD (ATRX-DNMT3-DNMT3L) domain also called the PHD (plant homeodomain) domain; 2) And a PWWP domain that is absent in *Dnmt3l*. The *Dnmt3l* protein also lacks the methyltransferase motifs IX and X and other catalytic residues that are found in the other DNA methyltransferases. The interacting partners and the functions of these domains are listed in Table 1.1.

There are many isoforms of *Dnmt3a* and *Dnmt3b* produced by alternative splicing. These isoforms differ in sizes and expression patterns however factors controlling this alternative splicing are not known. *Dnmt3a* produces two isoforms 1) Dnmt3A and 2) Dnmt3A2, which is shorter. The two proteins have similar methyltransferase activity but vary in their localization during development. DNMT3A has ubiquitous expression while, DNMT3A2 has a very specific expression pattern. DNMT3A2 is restricted to the ES cells and germ cells, suggesting that these two isoforms may have different tissue-specific functions in embryonic development (98). Like DNMT1, DNMT3 proteins are also regulated both at posttranscriptional and posttranslational levels (95).

Both in mice and in humans there are six isoforms of DNMT3B (96,121,122) that arise from alternative splicing and retain different portions of the N and C termini. Based on the retention of the active site in the C-terminus and *in vitro* activities, DNMT3B1 and DNMT3B2 proteins are catalytically active. DNMT3B3 and DNMT3B6 have been shown to be catalytically active *in vitro* (123) and *in vivo* (124). So far it is not known whether the other DNMT3B isoforms are active.

Because the DNA methyltransferase 3 isoforms are conserved in evolution it is likely that they have undiscovered functions, perhaps as regulators of the other catalytically active DNMT3Bs, or as modulators of the activity of other chromatin modifying proteins. It will be interesting to elucidate the molecular functions of the splice variants of these DNA methyltransferases in embryonic development in various model systems.

Complete ablation of *Dnmt1*, *Dnmt3a*, *Dnmt3b*, and *Dnmt3l* confers varying defects in embryonic development. (83,125-127). These defects have been summarized in Table 1.2. Although data from knock-out studies underscore the importance of these DNA methyltransferases during embryonic development, the early lethality of affected embryos renders them unsuitable to study the effects of these enzymes in later tissue-specific developmental programs.

1.7 DNA methylation in disease

Various human diseases have directly been related to defects associated with the DNA methylation machinery.

1.7.1 ICF syndrome

Type-1 ICF syndrome (***I***mmunodeficiency ***C***entromere instability and ***F***acial anomalies) is the only disease that is known to occur due to homozygous or compound heterozygous mutations in a DNA methyltransferase gene. The majority of mutations in DNMT3B are missense mutations, others include nonsense and splice-site mutations (64). ICF is a rare autosomal recessive

disorder affecting around 50 patients worldwide (65, 66). However, because the clinical symptoms are diverse and depending on the availability of proper health care the incidence may be poorly reported.

The ICF patients exhibit a 7% decrease in global 5mC levels (67). Based on mutations in DNMT3B, the ICF syndrome is divided into two classes. In the Type 1 ICF syndrome (65% of ICF patients) there are biallelic missense mutations in the C terminal catalytic domain of DNMT3B (28). Another study shows two related patients that exhibit mutations outside the catalytic domain primarily in the PWWP domain of DNMT3B (68).

In Type 1 ICF cases, patients suffer from decreased levels of humoral immunity despite the presence of B cells. The early stages of lymphocyte differentiation are not affected but there may be defects in lymphocyte maturation or activation (69). These patients exhibit a broad spectrum of facial anomalies that include hypertelorism (widely spaced eyes), micrognathia (small jaws), low-set ears, epicanthal folds and macroglossia (protrusion or enlargement of tongue), mental retardation, respiratory and intestinal tract infections and low birth weight (28).

In cytogenetic analyses, tandemly repeating, noncoding DNA regions like satellite 2 of the long arms of chromosome 1 and 16, and satellite 3 DNA in the long arms of chromosome 9, nonsatellite repeat NBL2 on acrocentric chromosomes and D4Z4 in subtelomeric regions of chromosomes 4 and 10 are demethylated in all tissues but chromosomal instability is associated only with lymphocytes (67, 70). In lymphocytes the juxtacentromeric heterochromatin of

chromosome 1, 9 and 16 is elongated leading to formation of multiradiate chromosomes (28). The hypomethylation of satellite 2 and 3 regions leads to centromeric instability (70).

The peripheral blood of ICF patients has only naïve B cells with defective negative selection and terminal differentiation (71). The chromosomes 1, 9 and 16 show branching after mitogen PHA (phytohemeagglutination) stimulation of lymphocytes.

However, in Type 2 ICF (nearly 40% of ICF cases), patients do not exhibit mutations in DNMT3B and these patients show the same phenotypes as the patients with this mutation. Type 2 patients also show hypomethylation of tandemly repeated DNA regions on the centromere called alpha satellites, in addition to hypomethylation of DNA regions seen in ICF type 1 (72, 73). Recently, type 2 ICF patients with mutations in a zinc-finger- and BTB (bric-a-bric, tramtrack, broad complex)-domain-containing 24 (ZBTB24) gene have been identified (74). Importantly, because of the lack of large patient sample sizes and smaller life spans of the affected patients, animal models that mimic the disease are required to dissect the molecular mechanism associated with the disease.

1.7.2 Imprinting defects

Genomic imprinting refers to the epigenetic mechanism that maintains gene repression in parental germ lines so that the progeny inherits monoallelic gene expression in parent-of-origin specific manner. There are a number of diseases that are associated with deregulated imprinting mechanisms; two of

them are Beckwith-Wiedemann syndrome (BWS) (75) and Prader-Willi/Angelman syndrome (PWS/AS) (76).

In BWS patients there is biallelc expression of IGF2, which in normal conditions is only expressed from the maternal allele. In PWS and AS there are deletions on chromosome 15 that affect the paternal allele in PWS patients and the maternal allele in AS patients. In some AS cases mutations have been mapped to UBE3A gene that is imprinted in a tissue-specific manner (77).

The imprinted genes have only one functional allele. If the imprinted gene has a tumor suppressor activity, then loss of heterozygosity (LOH) can cause cancer. Interestingly, a number of defects in imprinted genes are related to cancer predisposition due to LOH. These include neuroblastoma, acute myelogenous leukemia, rhabdomyosarcoma, sporadic osteosarcoma and Wilms' tumor (78).

1.7.3 Cancer

In cancer cells the global methylation picture is quite different from that in normal cells. At global levels the genomes of cancer cells are hypomethylated where as there is region specific hypermethylation leading to aberrant silencing of tumor suppressor genes and genes involved in all cellular pathways. There is little explanation for this paradoxical phenomenon. In a number of cancers this hypermethylation (29) is used as a prognostic marker.

However, it is still not clear why some genes are hypermethylated in certain forms of cancer while others are not. It is believed that DNA

hypomethylation leads to chromosomal instability that may lead to further mutations in genes thus indirectly causing cancer. In many cancers there is aberrant methylation of differentially methylated regions (DMRs) (15). One may speculate that insight into the interacting partners of the DNMTS may shed light into the aberrant recruitment of the DNMTS to different genetic loci in pathological conditions.

1.8 Conditional knockout models

To understand the involvement of DNA methyltransferases in tissue-specific methylation patterns different DNA methyltransferases have been conditionally knocked out alone or in combination. Also, ICF syndrome has been modeled in mice to dissect the molecular involvement of *Dnmt3b* in disease progression and pathophysiology.

1.8.1 ICF Syndrome

To model the ICF syndrome transgenic mouse lines were created carrying two missense mutations A609T and D823G that lead to partial loss of function of *Dnmt3b* (131). The missense mutant mice developed to term but most of the ICF neonatal mice died one day after birth. Some survived to adulthood. The mice that survived recapitulated only some aspects of the human disease. They displayed facial dysmorphism and low weight at birth. Unlike agamma-globulinemia that is observed in most patients these mice exhibit defects in the T cell lineage.

After birth, the numbers of thymocytes were reduced due to apoptosis in the thymus. This suggested that in mice *Dnmt3b* activity is essential for the survival of thymic T cells and not mature B cell populations. At the cytogenetic level the mice display hypomethylation of centromeric minor satellite DNA and juxtacentromeric major satellite DNA. Perhaps, differences in cytogenetics of DNMT3B deficient humans and mice arise due to differences in chromosomal organizations of these two organisms. In summary *Dnmt3b* deficient mice show more extensive DNA hypomethylation and the kind of immunological dysfunction is different from humans.

A challenge with modeling human diseases in animals is that most disease models only phenocopy certain aspects of the disease. Moreover, the extent of similarities may vary and some phenotyes may be completely model specific. However, to circumvent this caveat cumulative data from multiple model systems may be useful in generating a better understanding of a human disease. Importantly, because of the rare numbers of available ICF patient samples it is imperative to develop cost effective model systems that will recapitulate different aspects of the disease.

1.8.2 Conditional knockout of *Dnmt1*, *Dnmt3a* and *Dnmt3b* in HSCs

The hematopoietic system is a robust model system to study epigenetic decisions that regulate cell fate commitments (25, 132, 133). In a recent study Feinberg and colleagues mapped the methylation status of 4.6 million CpG sites

of murine hematopoietic progenitors (132). They identified differentially methylated genes of noncommitted and lineage-committed progenitors. This study identified lymphoid and myeloid specific genes like *Lck*, *Mpo*, *Cxxcr4* and *Gadd45a* that are regulated by promoter methylation in various stages of progenitor cell development.

Also, the authors further corroborated earlier findings which suggested that progression of uncommitted progenitors to the myeloid lineage requires less global DNA methylation than that needed for the generation of lymphoid lineage progenitors (134). Feinberg *et al.* (132) could faithfully recapitulate this observation *in vitro* by pharmacologically modulating CpG methylation. Treatment of bi-potential progenitors with 5-aza-2'-deoxycytidine increased myeloid progenitors at the cost of lymphoid progenitor cells. However, since 5-aza-2' -deoxycytidine is a nonspecific nucleoside inhibitor of the DNMTs (135), it cannot be distinguished whether this myelo-lymphoid lineage commitment is regulated discretely by *Dnmt1*, *Dnmt3a* or *Dnmt3b* or by a combination of these enzymes.

Interestingly, Feinberg *et al.* (132) also showed that at various stages of lineage commitment, the levels of *Dnmt3b* are dynamic. They are highest in multipotent progenitors. The levels of *Dnmt3b* first decrease, then again increase in later stages of lymphoid progenitors. In contrast, the myeloid progenitors show a progressive decrease of *Dnmt3b* transcript levels as they progress towards lineage restriction. Interestingly, this dynamic expression profile of *Dnmt3b* is itself regulated by promoter methylation. These epigenetically induced variations

in the levels of *Dnmt3b* during different steps of hematopoietic differentiation suggests that *Dnmt3b* may be required in a graded manner to maintain the chromatin states of hematopoietic genes involved in lineage commitment of blood cells.

However, in this study the authors did not investigate the levels of *Dnmt1*, *Dnmt3a* and *Dnmt3b* in terminally differentiated hematopoietic cells. Also, the question whether DNA methyltransferase function is required for the formation of terminally differentiated populations of blood cells has not been addressed to date. Answers to these questions are particularly relevant in the context of various hematological disease conditions that exhibit both mutations and transcriptional deregulation of the *DNMT* genes (28,136-138).

Different studies have implicated the involvement of the DNA methyltransferases in normal development of various hematopoietic cells. In a recent finding it was reported that during lymphocyte activation *Dnmt3a* expression is induced by T cell receptor stimulation. However, the protein levels of DNMT3A are reduced by costimulation (139). The exact functional consequences of this stage-specific transcriptional regulation of *Dnmt3a* in these two lymphocyte activation steps are not known. These authors also investigated the functions of DNMT3A using *Dnmt*3a knockout mice as a model system. Interestingly, they did not observe any defects in the maturation of T cells lacking DNMT3A.

Contrary to the above findings, other studies provide strong evidence of regulation of T cell differentiation by both *Dnmt3a* and *Dnmt3b*. Promoter

methylation is implicated in regulating the expression of *Foxp3*, a gene required for $CD4^+/CD25^+$ regulatory T cells, a cell type that plays a role in maintenance of immunological tolerance (140-143) in the periphery.

A SUMO E3 ligase PIAS1, binds to the *Foxp3* promoter to recruit DNMT3A and DNMT3B that confer promoter methylation. This promoter methylation negatively regulates the expression of *Foxp3* to prevent T cell differentiation into regulatory T cells (25). Interestingly, it has been reported that the demethylated and not the methylated regions of the *Foxp3* locus in regulatory T cells are bound by the *ETS1* protein to regulate the expression of *Foxp3* (144). *In vitro* experiments have identified physical interaction between the *de novo* DNA methyltransferases DNMT3A and DNMT3B and the ETS transcription factor PU.1, a key regulator of myeloid and B cell lineage development (103). Also, microRNA 29b (miR-29b) has been shown to regulate the expression of DNMT1, DNMT3A and DNMT3B in acute myeloid leukemia cells (145).

To further characterize hematopoietic functions of the DNMTs, investigators have employed tissue-specific loss-of-function approaches specifically targeting the hematopoietic compartment of model systems. Functional knockout of *Dnmt1* created by inducing the removal of the catalytic domain of DNMT1 in the HSCs is incompatible with survival (134). These mice die due to the inability of HSCs and the progenitors to differentiate and by the induction of apoptosis. The $Dnmt1^{-/chip}$ mice are produced on combining the hypomorphic *Dnmt1* allele with the *Dnmt1* null allele. It was observed that these

Dnmt1$^{-/chip}$ mice showed a severe reduction in the Lymphoid Primed Multipotential Progenitors and a 2.7-fold enrichment of long-term HSCs.

The *Dnmt1*$^{-/chip}$ mice exhibit a reduction in common lymphoid progenitors and T cell progenitors. Interestingly, the *Dnmt1*$^{-/chip}$ mice do not exhibit any changes in the frequencies of differentiated myeloid and erythroid cells. There are two possible explanations for this observation. First, DNA methylation may not be required for the formation of mature erythroid and myeloid cell populations. Second, instead of *Dnmt1*, *Dnmt3a* and/or *Dnmt3b* that are still expressed in the *Dnmt1*$^{-/chip}$ mice may regulate the differentiation of the myeloid and erythroid lineages into mature cells. Furthermore, hematopoietic progenitors in the bone marrow of the *Dnmt1*$^{-/chip}$ mice lack the expression of *Pax5*, an early B cell marker suggesting that decreasing the levels of *Dnmt1* indirectly silences *Pax5* expression. The authors also show the transcriptional upregulation and promoter demethylation of lineage-specific genes like *Gata1* and *Cd48* as a consequence of reduction in the levels of *Dnmt1* in the HSCs of *Dnmt1*$^{-/chip}$ mice.

In contrast to the above report, ablation of *Dnmt1* in the hematopoietic compartment, obtained by crossing *Dnmt1*$^{fl/fl}$ with interferon-inducible *Mx-Cre* transgenic mice, does not exhibit hematopoietic defects in terminally differentiated blood and progenitor cell types (146). This observation again alludes to the idea that *Dnmt1* may not be required for terminal differentiation of different lineages in hematopoiesis and that *Dnmt3a* and/or *Dnmt3b* may be involved instead. Based on cell transplantation studies and competitive transplantation assays these authors concluded that *Dnmt1* is not only required

for self-renewal and differentiation of the HSC cells but also for differentiation into mature myeloid cells only. These conclusions were primarily based on the observation that in competitive repopulation assays, unlike B and T cells, only the myeloid cells were drastically reduced. However, these defects could have arisen because of failure of competitive engraftments. Also, differences in the methods used for generating the transgenic lines in the studies described above may be responsible for the different outcomes.

Thus based on the data discussed above it is difficult to conclude whether *Dnmt1* is required solely for the generation of terminally differentiated cells of myeloid lineage (146) or for the lymphoid lineage (134) and also that *Dnmt3* family genes may play an unrecognized role in regulating formation of terminally differentiated hematopoietic cells. Further studies aimed at elucidating the functions of DNA methyltransferases are required to reconcile these observations and for dissecting the contributions of different DNMTs in various steps of hematopoiesis.

Because hypomorphic *DNMT3B* is involved in hematopoietic defects caused by ICF syndrome, the contribution of *Dnmt3a* and *Dnmt3b* was elucidated in hematopoiesis (147). In mice *Dnmt1*, *Dnmt3a*, *Dnmt3a2* and *Dnmt3b* are expressed at different levels in various populations of the hematopoietic cell types. *Dnmt3a*, *Dnmt3a2* and *Dnmt3b* are robustly expressed in B and T lymphoid lineages whereas their expression is very weak in neutrophil/macrophage and erythroid lineages. The CD34-/low, c-Kit+, Sca-1+,

lineage marker- (CD34- KSL) cells, a population enriched in hematopoietic stem cells has robust expression of all *Dnmt1*, *Dnmt3a*, *Dnmt3a2* and *Dnmt3b* (147).

Myeloid and lymphoid differentiation potential from HSCs were uncompromised when measured by *in vitro* colony forming assays, and by *in vivo* transplantation assays in mice lacking *Dnmt3a*, *Dnmt3b* or both in their hematopoietic compartment. However, when these HSCs were challenged with long-term reconstitution by transplantation assays they lost their reconstitution potential. The authors concluded that *Dnmt3a* and *Dnmt3b* are required for self-renewal but not for differentiation in hematopoietic stem cell (147).

Interestingly, different studies have suggested two different ideas regarding the contribution of *Dnmt1* during hematopoiesis. The first one claims that *Dnmt1* is solely required for the terminal differentiation of the myeloid lineage (134) and the second one suggests that it is only required for the generation of the terminally differentiated cells of the lymphoid lineage (146). However, further studies are required to address the requirement of the DNA methyltransferases during hematopoiesis.

DNA methylation has also been shown to play a role in the development of other organ systems. To study the contributions of DNA methylation in the central nervous system DNA methyltransferases have also been knocked out conditionally in the central nervous system (148-150).

1.9. Major unresolved questions

The data discussed above point out some unanswered questions in the field of DNA methylation and its impact on development and disease.

1. Do the different isoforms of DNMT3A and DNMT3B families have tissue-specific functions?

2. Do the different isoforms of DNMT3A and DNMT3B regulate each other as cofactors?

3. What are the functions of various DNMT3A and DNMT3B isoforms in imprinting and sex determination?

2. What are the contributions of the different *de novo* DNA methyltransferases in hematopoietic development?

3. What are the genetic targets of the different DNA methyltransferases?

4. What are the signaling pathways that are down stream effectors and regulators of DNA methyltransferases?

5. Can we establish tractable model systems for ICF syndrome?

6. Can we develop tractable model systems to screen for specific drugs against the different DNA methyltransferases?

Answers to the above questions are pertinent in understanding the contributions of different DNA methyltransferases in embryonic development and disease conditions. Also, they are essential for developing better therapeutics against the diseases discussed. To investigate the above queries we chose zebrafish as our model system.

1.10 Zebrafish as a model system

Danio rerio (zebrafish) is a popular model system for biological research. Briefly, zebrafish are easy and economical to maintain. They have short generation times. The females are highly prolific in producing eggs. Embryonic developmental patterns are similar to mammals (151, 152) and development is external and rapid thus providing easy access to clinical and genetic manipulations. Because the developing embryos are transparent, developmental processes can easily be monitored using standard techniques.

Notably, zebrafish are highly amenable to genetic (153) and pharmacological (154, 155) manipulations, this makes them tractable tools to study genetics and epigenetics (156). Lastly, the fact that the zebrafish genome is being sequenced renders them an attractive model system to dissect the roles of the evolutionary conserved DNA methyltransferases (157) in development (49, 60, 61, 158-162) and disease (163-166).

Based on sequence similarity, zebrafish harbor orthologs of all the mammalian DNA methyltransferases (167). The zebrafish DNA methyltransferase proteins bear resemblance both to the mammalian maintenance DNA methyltransferase *Dnmt1* (168,169) and the *de novo* DNA methyltransferases *Dnmt3a* and *Dnmt3b* (122).

1.11 DNA methyltransferases in zebrafish

Zebrafish harbor all known orthologs of mammalian DNA methyltransferases (167). Figure 1.5 shows the structures of the zebrafish DNA

methyltransferases. Zebrafish have seven DNA methyltransferase genes namely *dnmt1*, *dnmt3*, *dnmt4*, *dnmt5*, *dnmt6*, *dnmt7* and *dnmt8*. Based on sequence similarity and functional analyses it has been suggested that *dnmt1* is the maintenance DNA methyltransferase. Also, *dnmt3*, *4*, *5*, *6*, *7*, and *8* are homologs of mammalian *Dnmt3a* and *Dnmt3b*. Interestingly, both *dnmt1* mutant (158) and morphant (60) embryos largely phenocopy each other. Table 1.3 summarizes the zebrafish DNA methyltransferase morphant and mutant phenotypes.

Interestingly, zebrafish also harbor proteins that can actively demethylate exogenous methylated plasmids (49, 60) suggesting that zebrafish might utilize both DNA methylation and DNA demethylation to guide normal development. Notably, the lack of a protein that bears sequence similarity to *Dnmt3l* in zebrafish has lead to the idea that zebrafish do not have imprinting and provide a simple model system to study the contributions of different DNA methyltransferases in tissue-specific development.

Data from our lab suggests that *dnmt1* and *dnmt3* in zebrafish harbor both overlapping and discrete tissue-specific functions (60, 61). Based on the data discussed above, I hypothesize that *DNA methyltransferase 4*, *5*, *6*, *7* and *8* in zebrafish guide terminal differentiation of both common as well as discrete tissues during embryonic development.

1.12 Preview

While involvement of DNA methylation in regulating tissue-specific terminal differentiation has been a contentious issue. Recent data from our lab and others have supported this view. The data presented in this thesis characterize the phenotypic consequences of morpholino knock down of *dnmt4*, *dnmt5*, *dnmt6*, *dnmt7*, *and dnmt8*. These bear sequence similarity to the mammalian *Dnmt3a* and *Dnmt3b* family. Chapter 2 highlights the characterization of expression pattern, antisense morpholinos and impact of knock down on global methylation status of *dnmt4*, *dnmt5*, *dnmt6*, *dnmt7*, and *dnmt8*. Chapter 3 characterizes the details of molecular defects observed in *dnmt4* morphants. This chapter also delineates the requirement of *dnmt4* in zebrafish hematopoiesis. Also, in this chapter we show epistatic relationship between retinoic acid signaling and *dnmt4*. In Chapter 4 I have shown epistatic relationship between adenomatous polyposis coli (*apc*) and *dnmt4*.

In Chapter 5 I have summarized the main findings of this work and its implications. Appendix A shows the characterization of expression patterns, morpholinos, and morphological defects of methylated DNA binding proteins, a class of proteins that recognize methylated CpG dinucleotides. I have shown data for *mbd1*, *md3a* and *mbd3b* in zebrafish. Appendix B shows the effects of over expression of *dnmt4* on blood cells.

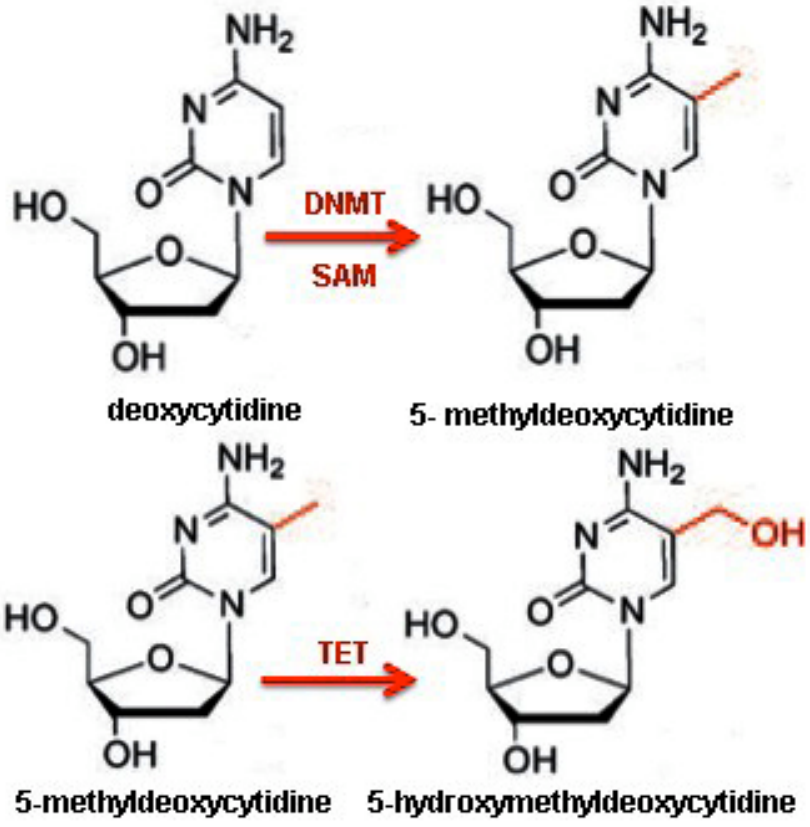

Figure 1.1. The two main modified nucleotides present in DNA of mammals. Cytosine on deoxycytidine (present in CpG dinucleotide context) are modified to 5-methyldeoxycytidine by DNA methyltransferases that use s-adenosylmethionine (SAM) as a cofactor. The 5mC is often called the 5th nucleotide. 5-methyldeoxycytidine can further be modified into 5-hydroxymethyldeoxycytidine by TET proteins. The 5-hydroxymethylcytosine (5hmC) is referred to as the 6th nucleotide. These two modified nucleotides have roles in regulating gene expression.

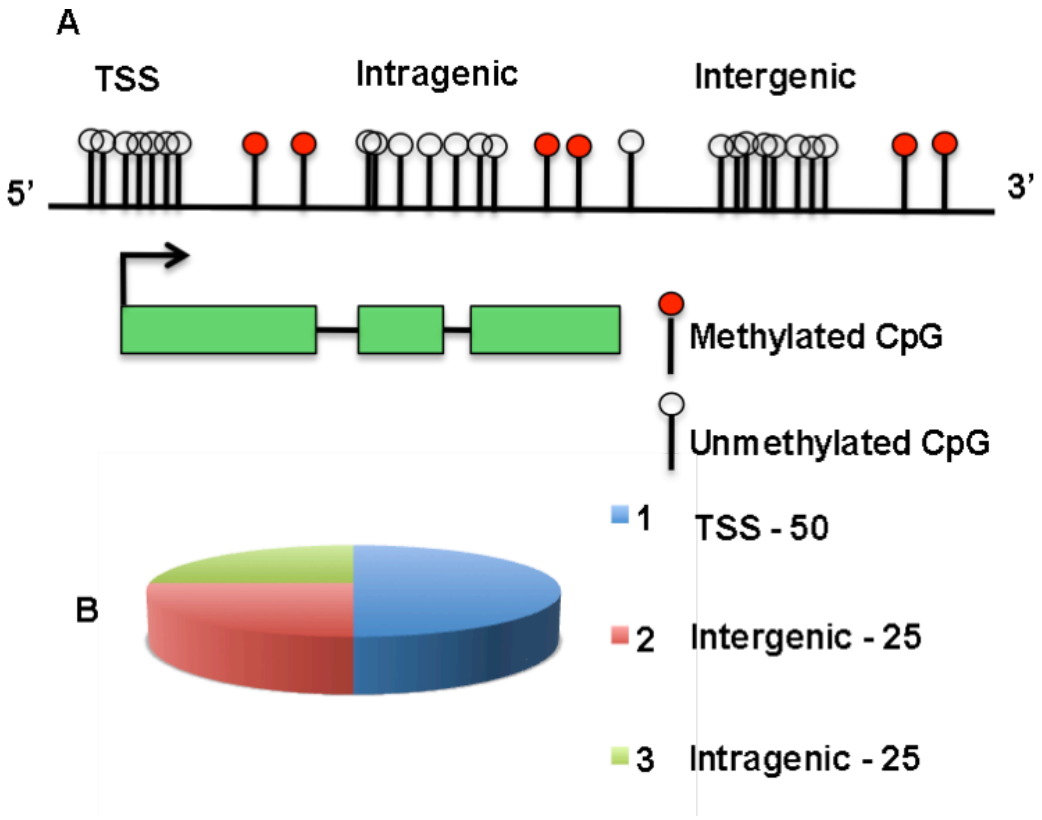

Figure 1.2. The genomic distribution of CpG dinucleotides. (A) In the genomes of higher eukaryotes the distribution of CpG dinucleotides is non random. Regions where CpG dinucleotides are expressed at their statistically expected frequency are called CpG islands. The CpG islands are present in the transcriptional start sites (TSS), intragenic regions and intergenic regions. (B) The pie chart shows the distribution of CpG islands as a percentage in the human genome. 50% of the CpG dinucleotides are present in the start sites (TSS), and 25% each in the intragenic regions and intergenic regions. The total number of genes analyzed was 25,495. The data in (B) is adapted from Illingworth et al., 2010.

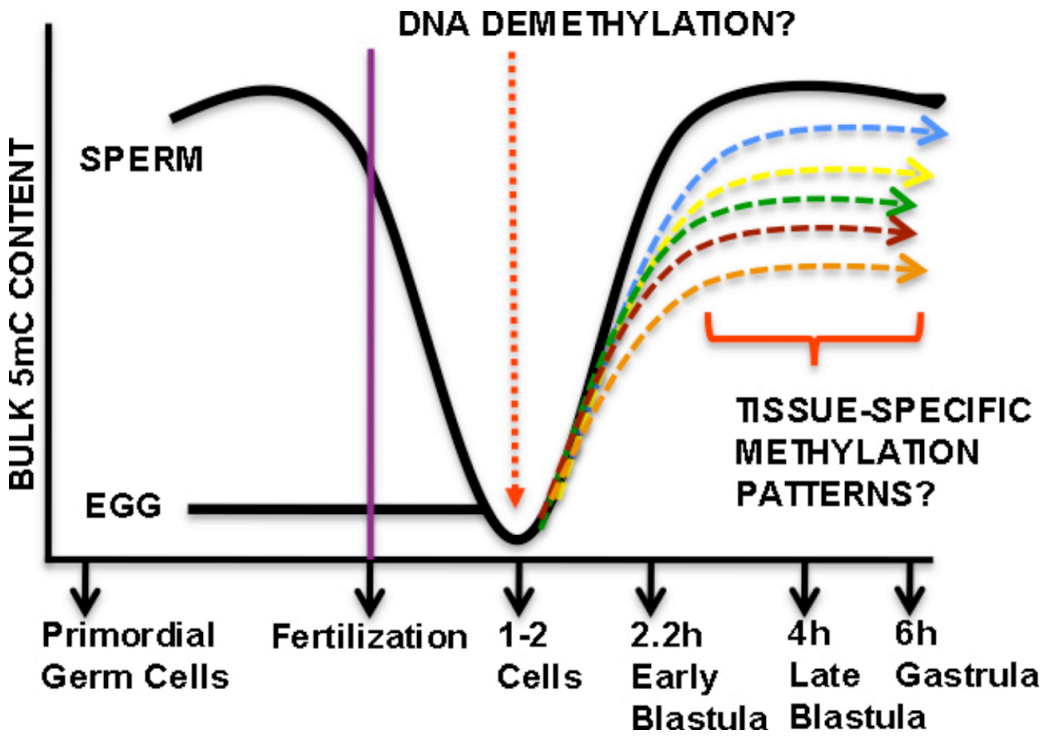

Figure 1.3. Dynamic profiles of bulk methylation in development

The dynamic profiles of bulk levels of 5mC in the genome of the developing embryo and in the primordial germ cells. The global levels of bulk methylation are higher in sperm in comparison to the egg. At fertilization the bulk methylation levels of the zygote first decrease, suggesting the occurrence of active DNA demethylation and then are regained in later stages of embryonic development suggesting that this remethylation may occur in a tissue specific manner to confer cell identity and terminal differentiation**.** However, it is not known which of the DNA methyltransferases target the different genetic loci during this tissue specific remethylation phase. This figure is adapted from Heby et al., 1995, Mhanni et al., 2004 and Rai et al., 2008.

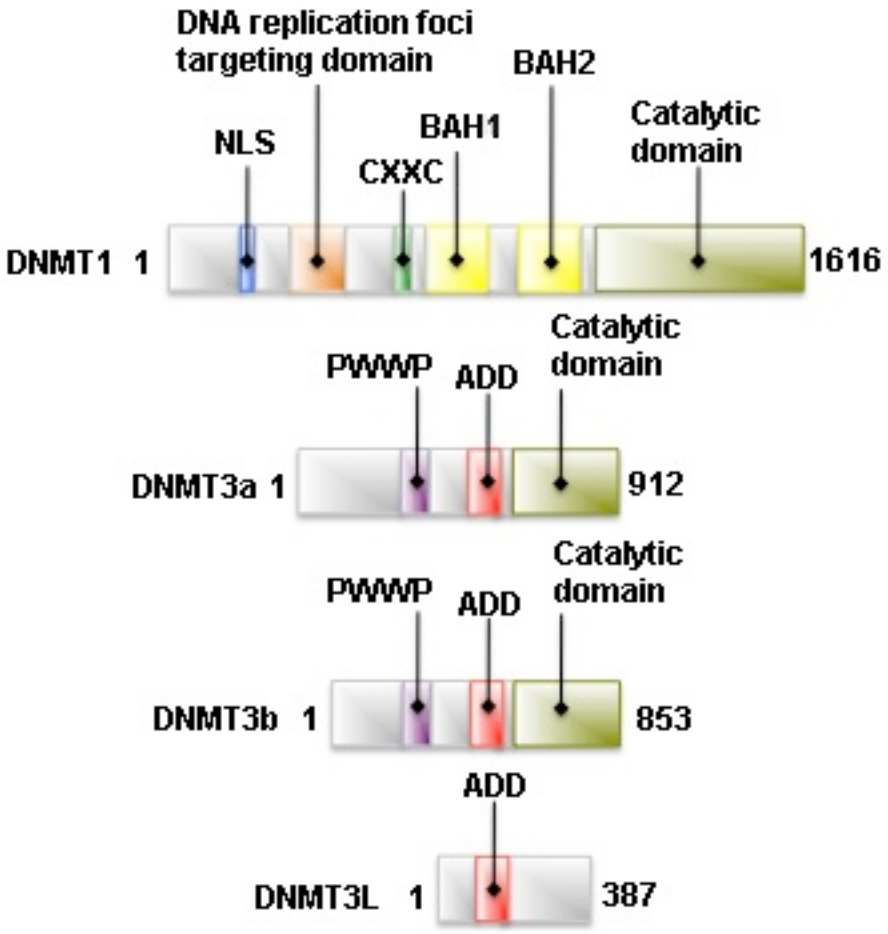

Figure 1.4. Structure of the mammalian DNA methyltransferases. The schematic of different DNA methyltransferases in mammals and their domain structures. DNMT1 is the primary maintenance DNA methyltransferase and DNMT3A, DNMT3B and DNMT3L represent the main *de novo* members. The DNA methyltransferases harbor a highly conserved C-terminal catalytic domain and a divergent N-terminal regulatory domain. Perhaps the N-terminus may help in recruiting different DNMTs to their genetic targets.

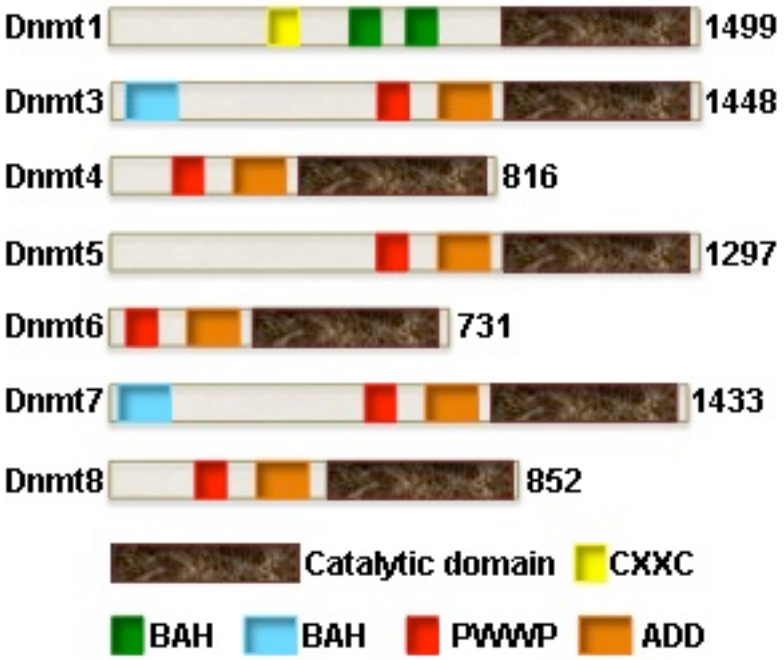

Figure 1.5. Diagram of the DNA methyltransferases in the zebrafish.

Zebrafish harbor both the maintenance and the *de novo* DNA methyltransferases in their genome. They have a conserved C-terminal catalytic domain and a divergent N-terminal region. The different domains in the N-terminal help in protein-protein interactions and may help in targeting the proteins to their genetic loci. We hypothesize that different zebrafish DNA methyltransferases may be targeted to different tissues during the re-methylation phase during embryonic development to confer tissue-specific epigenetic reprogramming.

Table 1.1. Interacting partners of DNA methyltransferases.

DNMT1	**Ref.,**	**DNMT3A**	**Ref.,**	**DNMT3B**	**Ref.,**
HDAC1	(99)	HDAC1	(100)	HDAC1	(101)
DMAP1	(102)	PML-RAR	(103)	DNMT3L	(104)
HDAC2	(102)	MYC	(105)	SUMO-1	(106)
PCNA	(107)	RP58	(100)	UBC9	(106)
Rb	(108)	SUV39H1	(109)	DNMT3A	(104)
E2F1	(110)	HP1	(109)		
MECP2	(111)	PIAS1	(112)	**DNMT3L**	
DNMT3A	(97)	UBC9	(112)	HDAC1	(113)
DNMT3B	(97)	PIASxa	(112)	DNMT3A	(104)
SUV39H1	(109)	DNMT3L	(104)	DNMT3B	(104)
SET7/9	(114)	PU.1	(103)		
G9A	(115)	SETDB1	(116)		
EZH2	(117)	EZH2	(117)		
UHFR1	(118)	MBD3	(119)		
PML-RAR	(120)	BRG1	(119)		
HP1	(109)				

Table 1.2. Phenotypes of murine knockouts of DNA methyltransferases.

Gene (References)	Mutant phenotype	DNMT activity
***Dnmt1*$^{-/-}$ (125)**	- Embryonic lethality (E8.5) - Genome wide hypomethylation - Loss of imprinting	- YES - Main maintenance DNMT - *de novo* activity – Yes, low
***Dnmt3a*$^{-/-}$ (83)**	- Post natal lethality (4 weeks) - Loss of *de novo* methylation - Severe defects in gut and spermatogenesis	- YES - *de novo* activity, - some maintenance activity
***Dnmt3b*$^{-/-}$ (83)**	- Developmental arrest (E14.5-E18.5) - Loss of *de novo* methylation - Mild neural tube defects - Demethylation of minor satellite repeat sequences	- YES - *de novo* activity; - some maintenance activity - Preference for minor satellite repeats
***Dnmt3l*$^{-/-}$ (128-130)**	- Viable; males are sterile (spermatogenic defects) - Females have no viable progeny - Loss of maternal and paternal imprints	- NO - Cofactor of DNMT3A (enhances its *de novo* activity)

Table 1.3. Zebrafish dnmt mutant and morphant phenotypes.

Gene	Mutant	Morphant	Phenotypes/Reference
dnmt1	dandelion	Yes	Loss of global methylation, curled tails, pericardial edema, defects in jaws, intestine, retina, exocrine pancreas (60, 158)
dnmt3	No	Yes	Small brains, defective pharyngeal arch formation, defective retinal neural epithelial differentiation (61)
dnmt4	No	Yes	No (167)
dnmt5	No	Yes	No (167)
dnmt6	No	Yes	No (167)
dnmt7	No	Yes	No (167)
dnmt8	Yes	Yes	No (167)

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CHAPTER 2

CHARACTERIZING *DE NOVO* DNA METHYLTRANSFERASES IN ZEBRAFISH

2.1 Introduction

Danio rerio or zebrafish is a widely used model system to study development (1, 2) and disease (3, 4). Because murine knockout models of critical genes are embryonic lethal they are not useful in studying later differentiation programs. Zebrafish offers an evolutionary conserved, cost effective, prolific and tractable model system to study contributions of genes in normal development and diseases. Large-scale mutagenesis screens in zebrafish have identified mutants that phenocopy human diseases thus consolidating the usefulness of this model system in disease related research.

Also, injection of antisense morpholinos leads to formation of hypomorphic zebrafish embryos that have subtle developmental defects in comparison to the control embryos. The morpholino knockdown can also lead to a wide range of phenotypic and molecular defects. However, specific and nonspecific defects of morpholino induced knockdown can be distinguished using widely acceptable criteria. These amenable properties of zebrafish circumvent the problems of embryonic lethal phenotypes observed in murine knock out models.

In zebrafish a total of eight genes that bear sequence homology to eukaryotic DNA methyltransferases have been described. These are *dnmt1*, *dnmt2*, *dnmt3*, *dnmt4*, *dnmt5*, *dnmt6*, *dnmt7* and *dnmt8* (Figures 1.5 and 2.7) (5). However, unlike mammals which possess three distinct DNMT3 family members zebrafish harbors six proteins which resemble in sequence either the mammalian DNMT3A or DNMT3B (5). Importantly, of the six DNA methyltransferases that bear sequence homology to mammalian DNA methyltransferases there is none that resembles DNMT3L.

Interestingly, both the mouse and human genomes harbor six isoforms of DNMT3B that arise due to alternative splicing (6-8). Given this conservation it is possible that these isoforms might have specific biological functions. However, this possibility has not been investigated although there is evidence that some of these splice variants are enzymatically active (9, 10) and are deregulated in certain cancers (11, 12).

The six members of the Dnmt3 family in zebrafish namely *dnmt3*, *dnmt4*, *dnmt5*, *dnmt6*, *dnmt7* and *dnmt8* contain a conserved C-terminal catalytic domain and an N-terminal regulatory domain that harbor the characteristic PWWP (pro-try-trp-pro) and ADD (ATRX-DNMT3-DNMT3L) motifs (Figures 1.4 and 2.7).

Zebrafish *dnmt6* and *dnmt8* are orthologous to DNMT3A. The sequence identity between *dnmt6* and *dnmt8* is significant with 77% over all identity and 89% identity in their catalytic domains (13). However, it is still not clear whether these two proteins have distinct and/or overlapping expression patterns and

functions during development.

Because of the differences in the N-terminal regulatory domains it is conceivable that these two genes in zebrafish may have distinct embryonic functions. Moreover, the two isoforms of the mammalian DNMT3A (DNMT3A and DNMT3A2) have distinct expression patterns.

DNMT3A is ubiquitously expressed in all tissues and DNMT3A2 is expressed in embryonic stem cells, germ cells, and embryonal carcinoma cells. While it is not expressed in adult tissues, but some expression is observed in spleen and thymus (14). Zebrafish is an amenable model system to investigate the tissue-specific functions of homologs of DNMT3A and DNMT3A2 in development.

Zebrafish genes *dnmt3*, *dnmt4*, *dnmt5*, and *dnmt7* also bear sequence homology to the mammalian DNMT3 proteins (5). Dnmt4 exhibits 68% amino acid sequence identity with the mouse DNMT3B (13). Importantly, mutations in the human DNMT3B leads to immunodeficiency, centromere instability and facial anomalies (ICF) syndrome (15). However, whether *dnmt4* regulates immune functions like human DNMT3B and is involved in hematopoietic programs is still not known (5).

The remaining members *dnmt3*, *dnmt5*, and *dnmt7* are fish-specific DNMT3 family members and their sequence does not suggest higher similarity to either DNMT3A or DNMT3B (13). Zebrafish *dnmt3*, *dnmt4*, and *dnmt5* are present in the same gene cluster (16), but it is still not known if they have similar or discrete molecular functions during embryonic development.

The recent discovery of an active DNA demethylation machinery in zebrafish (17) suggests that like mammals, zebrafish might use methylation and demethylation to regulate epigenetic reprogramming. This further underscores the utility of this nonmammalian vertebrate in studying the impacts of DNA methylation in development and disease. These data suggest that zebrafish offers a unique opportunity to dissect possibilities of tissue-specific functions of various Dnmt3 isoforms in embryonic development.

To this end we have analyzed the expression patterns of zebrafish *dnmt4*, *dnmt5*, *dnmt6*, *dnmt7* and *dnmt8* at different stages of embryonic development. Further we have characterized antisense morpholinos for each of these genes to examine the gross morphological phenotypes and the impact of depletion of these enzymes on global methylation levels during embryognesis.

Such analyses will be useful in screening novel DNA methyltransferase inhibitors that are specific against different DNA methyltransferases. An important problem associated with the current DNA hypomethylating drugs is that they are not specific against any particular DNA methyltransferase thus causing unwarranted side effects. Zebrafish offers an economic and useful system to test the specificity of different DNA hypomethylating drugs against different isoforms of *dnmts*.

In this study we have characterized the embryonic expression patterns of *dnmt4*, *dnmt5*, *dnmt6*, *dnmt7* and *dnmt8* that are orthologs of either mammalian Dnmt3A or Dnmt3B in zebrafish. Based on previous data from our lab I hypothesize that *dnmt4*, *dnmt5*, *dnmt6*, *dnmt7* and *dnmt8* are *de novo* DNA

methyltransferases in zebrafish and are required in a tissue-specific manner to guide organ development.

2.2 Materials and methods

2.21 Zebrafish maintenance

Zebrafish stocks were maintained in z-mods on a 14:10 hour light:dark cycle at 28°C in the Hunstman Cancer Institute zebrafish fish facility using standard fish husbandry IACUC protocols. Fertilized embryos were grown at 28.5°C. For whole mount *in situ* hybridization embryos were raised in 0.003% phenylthiourea to inhibit pigment formation.

2.22 Zebrafish microinjections

Morpholino injections were done at 1-4 cell stage. Morpholinos were ordered from Gene Tools LLC. Morpholino oligonucleotides and the splice junctions they were designed against for *dnmt4*, *5*, *6*, *7*, and *8* are shown in Table 2.1.

2.23 Oligonucleotides and constructs

The GenBank accession numbers for *dnmt4*, *5*, *6*, *7*, and *8* are NM_001025450, NM_001020479, NM_001018140, NM_001020476 and NM_001018134 respectively. Zebrafish *dnmt4*, *5*, *6*, *7* and *8* were cloned in pCRTOPOII vector. Splice check was performed using the primer sets shown in Table 2.1.

2.24 Whole mount *in situ* hybridizations

Zebrafish embryos at mentioned time points were fixed in sucrose buffered 4% para-formaldehyde. The embryos were washed in PBS, dehydrated and stored in methanol at -20°C. Whole mount *in situ* hybridizations were carried out as described previously (18) using digoxigenin-labeled riboprobes for *dnmt4*, *dnmt5*, *dnmt6*, *dnmt7*, and *dnmt8*.

2.25 Global DNA methylation assay by LC-MS

Embryos were collected at 24hpf and 72hpf and genomic DNA was isolated using Gentra Systems PURGENE DNA Purification kit. Global DNA methylation analysis was performed using liquid chromatography-mass spectrometry as described previously (20).

2.26 Statistical analyses

Prism 4 (GraphPad Software, San Diego, CA) was used for statistical analyses. The quantitative data are representative of at least three independent experiments.

2.3 Results

To determined the dynamics of changes in the bulk DNA methylation levels in zebrafish development we used a mass spectrometry-based assay (20), (Figure 2.1) and observed an approximately 80% decrease in global methylation

levels of the 1hpf zebrafish embryo (mdC/dG content of 1.94% ± 0.32% (ave ± S.D.) in comparison to the 24hpf embryo (mdC/dG content of 6.89% ± 0.36% (ave ± S.D.). The global methylation levels of the whole adult fish is nearly the same as the 24hpf embryo: 6.74% ± 0.51% (ave ± S.D.). This difference in the genomic methylation levels of the 1hpf embryo and the 24hpf embryo suggest that during development of the newly fertilized embryo active DNA methylation must be occurring in the genome. However, it is not known if this methylation occurs in tissue-specific patterns. Also, which of the DNA methyltransferases are actively involved in conferring this *de novo* methylation are not known. It will be interesting to determine how early in embryonic development these bulk methylation patterns are re-established. Importantly, because the bulk methylation levels are representative of the silenced repetitive elements in the fish genome they cannot be used to elucidate the methylation dynamics at promoters of genes in different tissues.

A different approach needs to be employed to elucidate tissue-specific methylation patterns in embryonic development. The promoter methylation of genes in pure populations of different kinds of cell types from different organs needs to be used. Various techniques, such as bisulfite sequencing of target gene promoters, whole genome bislufite sequencing, and Methylated DNA immuno precipitation (MeDIP) and promoter array hybridization (aCGH) analyses can be performed.

To determine where *dnmt4*, *5*, *6*, *7*, and *8* expressed in the zebrafish embryos we performed *in situ* hybridization analyses. Interestingly, *dnmt4*, *5*, *6*,

7, and *8* are expressed in a tissue-specific manner. The expression is observed both in common as well as discrete organs. At earlier stages e.g. 24hpf the expression of the *dnmt4* transcript is ubiquitous in the anterior structures such as different parts of the brain, immature eyes, pharyngeal arches skeleton, and pectoral fin musculature. In the posterior structures *dnmt4* expression is confined to the hematopoietic compartment, such as the artery and certain populations of blood cells (Figure 2.2).

By 72hpf the expression of *dnmt4* becomes tissue-restricted and is robustly expressed in the tegmentum, telencepalon, diencephalon, ventricular zone, retina, pharynx, gut, pectoral fin, pharyngeal arches, artery and hematopoietic cells. These data are consistent with a previous report (21) that shows strong expression of murine *Dnmt3b* in the hematopoietic stem cells and uncommitted progenitor cells. Importantly, Dnmt4 exhibits 68% amino acid sequence identity with mouse DNMT3B (13). Despite the strong homology between *dnmt4* and *Dnmt3b,* morpholino knockdown of zebrafish *dnmt4* did not confer any phenotypic defects (5).

Dnmt5 is a ubiquitously expressed gene that is maternally supplied (Figure 2.3). At 24hpf and 36hpf the *dnmt5* gene is robustly expressed in a number of tissues including the brain, musculature, eyes, and the notochord suggesting it may be required for early stages of development. At around 72hpf the expression pattern of *dnmt5* becomes very localized to the pharyngeal arches, jaws, intestine, pectoral fins, brain and the eyes. It is also expressed in a subpopulation of blood cells at 96hpf.

Dnmt6 transcripts in the embryos are also maternally supplied (Figure 2.4). The expression of *dnmt6* at 24hpf is ubiquitous. However, by 48hpf the expression of *dnmt6* becomes localized more in the anterior regions of the embryos particularly the brain, eyes and the fins, with some expression in the gut. Interestingly, *dnmt6* appears to be expressed in the neural crest cells. This pattern of expression is consistent with the requirement of *Dnmt3a* in neuronal development (22).

Dnmt7 in zebrafish is also maternally supplied like the rest of the DNA methyltransferases. The expression of *dnmt7* is very ubiquitous in early development. In late stages it is quite prominent in the brain, eyes, pharyngeal arches, pectoral fins and the gut. This is contrary to a previous report (23) that suggests that at 48hpf there is restricted expression of *dnmt7*. Interestingly, *dnmt7* appears to be expressed in a subpopulation of blood cells at 48h. However, at 72hpf the expression does not seem to be as robust in the blood cells (Figure 2.5). It has been reported that *dnmt7* methylates the *ntl* gene (5). However, the authors did not report if this methylation regulates promoter activity of *ntl.* Interestingly, these authors did not find any phenotypic defects in the *dnmt7* morphants.

The expression pattern of *dnmt8* at different time points is shown in Figure 2.6. *Dnmt8* is expressed ubiquitously at early time points particularly in the neural tissues and the pronephric ducts. It is expressed in the somites, brain, the pronephric ducts, eyes, brain, pharyngeal arches and gut at 72hpf, 84hpf, and 96hpf.

A previous report has suggested that knock down of *dnmt4, dnmt5*, *dnmt6*, *dnmt7* and *dnmt8* does not confer any gross morphological phenotypes in the morphants (5). Interestingly, the authors used translation-blocking morpholinos for their analyses, but did not verify the knock down of the protein levels because of lack of *dnmt4, dnmt5*, *dnmt6*, *dnmt7* and *dnmt8* antibodies.

Taking into consideration this lack of resource we employed a different approach by designing splice-blocking morpholinos for different splice junctions of the DNA methyltransferase genes. The antisense splice blocking morpholinos were designed for splice junctions in the N-terminus of the enzymes. A diagram of the zebrafish DNA methyltransferases showing the various domains in the N-terminus is represented in Figure 2.7. The design of each morpholino and the junctions used is also represented in Figure 2.7.

Splice blocking morpholinos were injected into 1-4 cell stage embryos. Different morpholinos injections lead to different levels of survival in the morphants (Figure 2.9). The data from three separate experiments is plotted in this graph. Interestingly, *dnmt4*, *dnmt5* and *dnmt7* morphants showed decreased survival in comparison to the control injected embryos – 51%, 53% and 55% survival respectively in comparison to 79% 80%, and 93% survival of the control morphants. Verification of morpholino effect on splicing was performed at 24h. The reduction in spliced product for *dnmt4, dnmt5*, *dnmt6*, *dnmt7*, and *dnmt8* and the formation of unspliced product is represented in Figure 2.8.

The five *dnmt* morphants showed different gross morphological defects. *dnmt4*, *dnmt5*, *dnmt6*, and *dnmt7* morphants displayed pericardial edema. Tail

curvature defects were seen in *dnmt5* and *dnmt7* morphants. Prominent microphthalmia or small eyes were observed in *dnmt4* and *dnmt7*. Interestingly, *dnmt4* morphants displayed a lack of yolk sac extension and a globular yolk (Figure 2.10). Importantly, to determine the requirement of different *dnmts* in embryonic development it will be necessary to characterize what genes are affected in these morphants.

To determine if the *dnmt4, dnmt5*, *dnmt6*, *dnmt7* and *dnmt8* are indeed *de novo* DNA methyltransferases we performed LC-MS quantification of dC/dG content on the genomic DNA of these morphants at 24hpf and 72hpf. As, expected we did not observe appreciable changes in the levels of bulk methylation in the morphants and the controls Figure 2.11

2.4 Discussion

Previous reports from our lab have demonstrated that zebrafish *dnmt1* is expressed in proliferative tissues like retina, parts of the brain, pharyngeal arches and the gut (24). Morpholino-induced knockdown of *dnmt1* leads to terminal differentiation defects of the pancreas, retina and the gut in 96hpf embryos.

However, the progenitor specification of these organs is unaltered, suggesting a role for *dnmt1* in regulating terminal differentiation of the eyes, gut and pancreas (24). Importantly, knockdown of *dnmt1* conferred 40% reduction in global genomic methylation levels (24) suggesting that like the mammalian derivative, zebrafish *dnmt1* is a maintenance DNA methyltransferase.

Dnmt1 protein is 73% identical with the human DNMT1 and it's C-terminal

conserved catalytic domain has a 89% identity to the human protein (13). Complementation of the human derivative rescued the defects in *dnmt1* morphants, suggesting that *DNMT1* might function in similar tissues. Recent findings from *dnmt1* mutant lines in zebrafish *(26)* are in agreement with the molecular phenotypes observed in morpholino-induced knock-down studies (24).

Interestingly, zebrafish is the only organism in which transient knockdown of Dnmt2 reveals roles for this protein in embryonic development. Unlike the other family members of the zebrafish DNA methyltransferases Dnmt2 is an RNA methyltranferase (27). This is in agreement with the RNA methylating functions of mammalian DNA methyltransferases (28-31). This finding strongly supports the use of zebrafish as developmental model. The *dnmt2* morphants harbor defects in terminal differentiation of the embryonic retina, liver and brain at 80hpf (27), indicating that zebrafish is a useful vertebrate model system to study tissue ontogeny.

dnmt3 morphants exhibit terminal differentiation defects in the brain while the specification and patterning of the brain was unaffected. This highlights the functions of this gene in tissue-specific terminal differentiation (19). Moreover, in this study we have also demonstrated that in zebrafish, *dnmt1* and *dnmt3* cannot complement for the loss of each other although both the proteins are expressed in the gut and the brain. This suggests that in spite of redundant expression *dnmt1* and *dnmt3* have tissue-specific functions.

The absence of a protein that resembles in sequence to Dnmt3l propagated the idea that zebrafish lack imprinting (33). Also, this idea finds

support in the fact that diploid androgenote and gynogenote zebrafish can survive to adulthood and are fertile, suggesting that monoallelic expression by imprinting likely does not occur in a developmentally regulated manner in zebrafish (34, 35).

However, another report indicates that there are parent-of-origin effects on methylation of transgene in zebrafish (36). Furthermore, transposable elements (sequences methylated by DNMT3L in mice) are known to be methylated in zebrafish (37). To date, the proteins that are involved in this phenomenon have not been identified in zebrafish. A thorough functional approach must be employed to identify functional similarities between zebrafish DNA methyltransferase proteins and the mammalian homologs to identify proteins and interacting partners that may be involved in imprinting or similar regulatory processes.

Although *dnmt4*, *dnmt5*, *dnmt6*, *dnmt7*, and *dnmt8* have redundant expressions (as shown in Figures 2.2-2.6) they may have non-redundant functions depending upon various regulatory mechanisms that aid in activation or repression of the DNA methylatransferase (39, 40). In support of this idea we show that morpholino knockdown of different DNA methyltransferases confers different gross-morphological defects in the embryos (Figure 2.10). These differences may arise because the *dnmts* may affect different genetic targets. However, a detailed comparative analysis of the genetic targets needs to be performed to support this idea.

Also, our data indicates *dnmt4*, *dnmt5*, *dnmt6*, *dnmt7*, and *dnmt8* in

zebrafish function as *de novo* DNA methylatransferases as seen by lack of changes in global DNA methylation levels in the morphants. This indicates that zebrafish *dnmt4*, *dnmt5*, *dnmt6*, *dnmt7*, and *dnmt8* are functionally similar to mammalian Dnmt3a or Dnmt3b. However, functional assays need to be performed to determine the homology between the fish and the mammalian proteins. In this study we have characterized the expression patterns, and morpholinos for zebrafish *dnmt4*, *dnmt5*, *dnmt6*, *dnmt7*, and *dnmt8*. Because zebrafish dnmts are orthologous to mammalian DNA methyltransferases, zebrafish can be used to screen for specific drugs against different DNA methyltransferases.

2.5 Acknowledgements

Smitha R. James performed the global genomic 5mC analyses by LC-MS quantification.

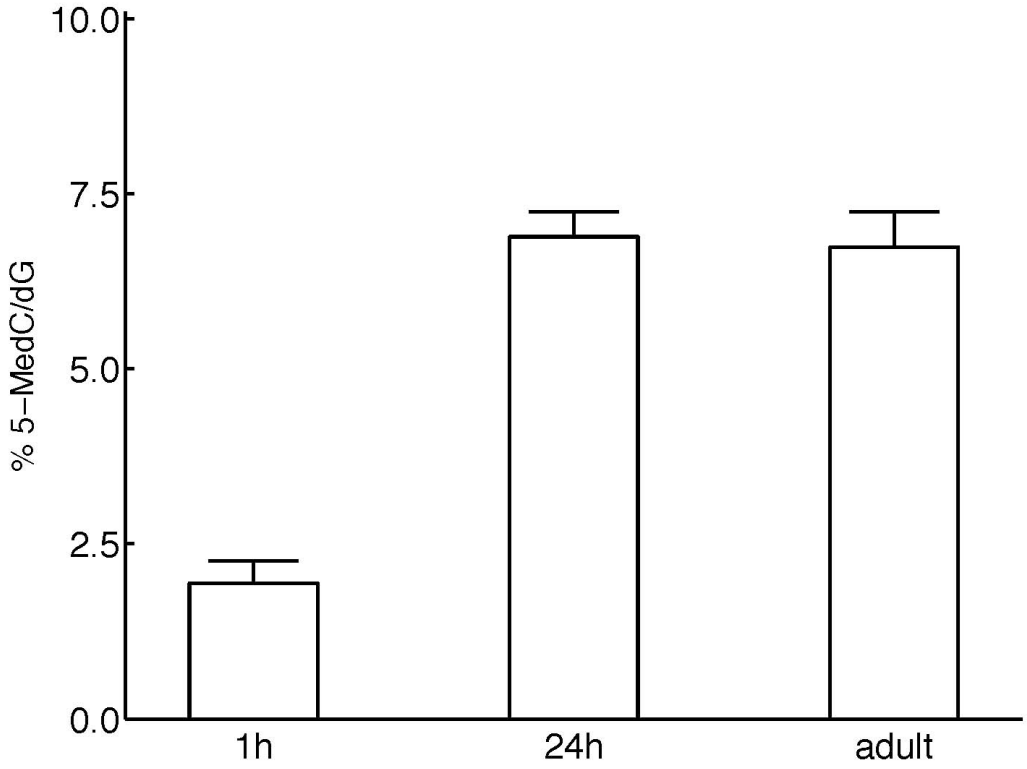


Figure 2.1. Dynamic changes in the bulk methylation levels of the zebrafish genome during development. LC-MS quantification was performed to analyze the bulk %5mdC/dG content at different stages of embryonic development. The newly formed zygote at 1hpf has a much lower bulk methylation than the 24hpf embryo. It is not known whether during the remethylation of the genome there is establishment of tissue-specific methylation patterns.

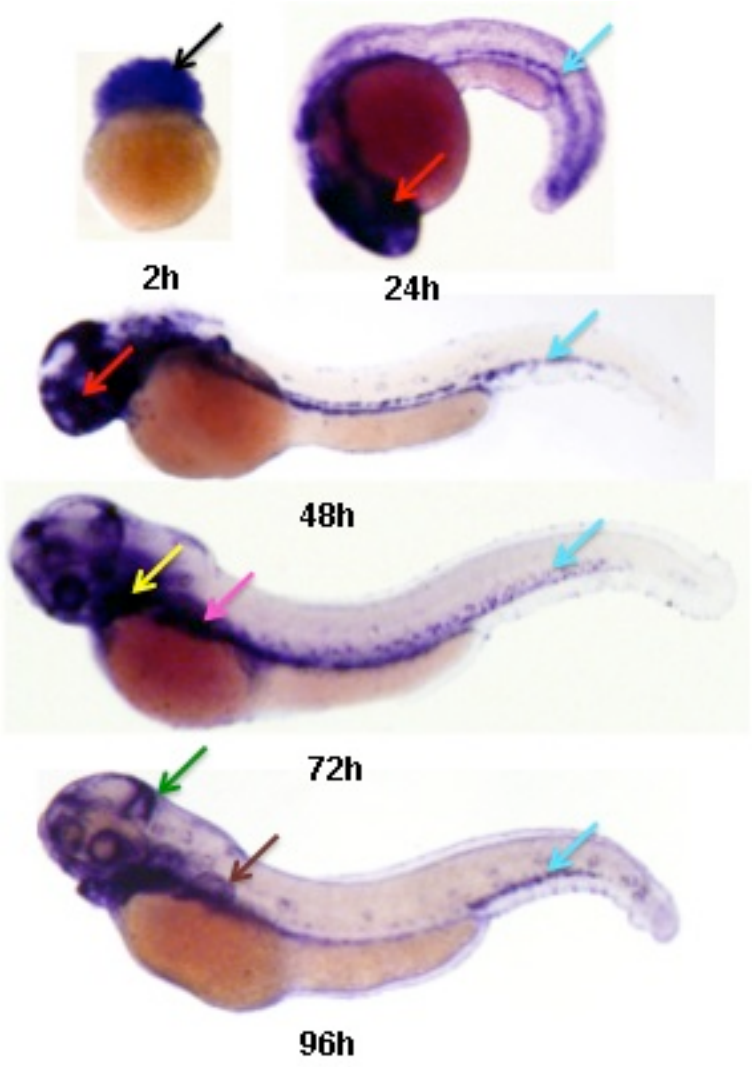


Figure 2.2. Expression pattern of *dnmt4* during embryonic development.

Zebrafish *dnmt4* is a maternally supplied transcript (black arrow) and is expressed in the eyes (red arrow), brain (green arrow), fins (brown arrow), pharyngeal endoderm (yellow arrow), gut (pink arrow) and a subpopulation of blood cells (blue arrows).

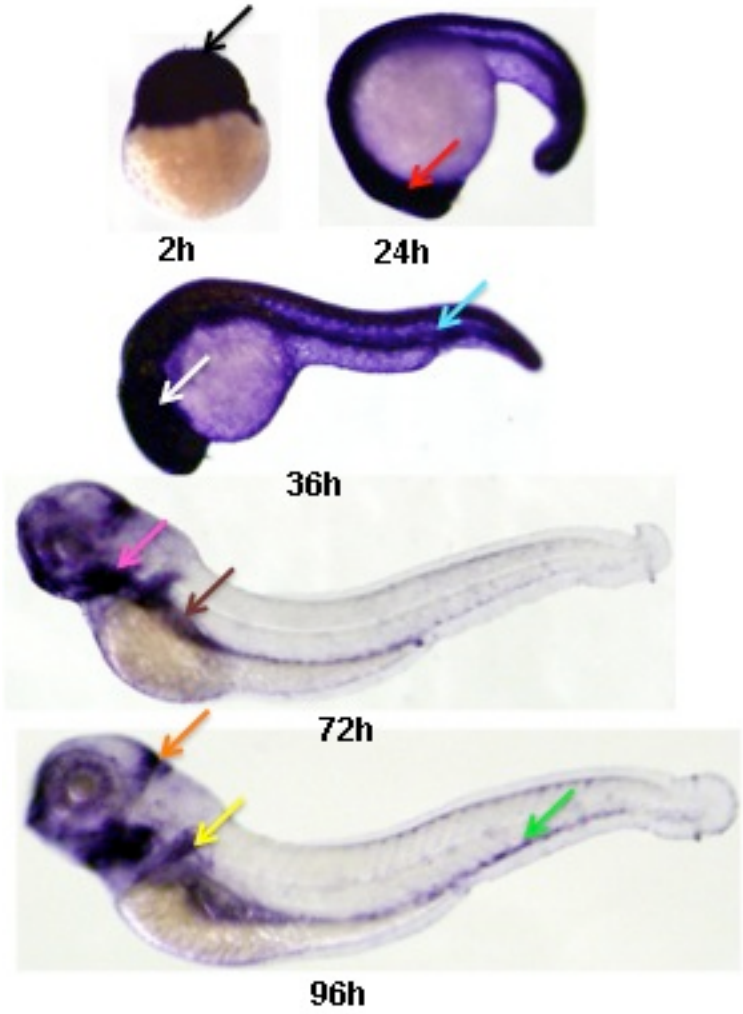


Figure 2.3. Expression pattern of *dnmt5* during embryonic development.

Zebrafish *dnmt5* is a maternally supplied transcript (black arrow). *dnmt5* is expressed in the eyes (red arrow) brain (white arrow), fins (yellow arrow), pharyngeal endoderm (pink arrow), gut (brown arrow) and a subpopulation of blood cells (green arrow). It is robustly expressed in the pronephric duct (blue arrow).

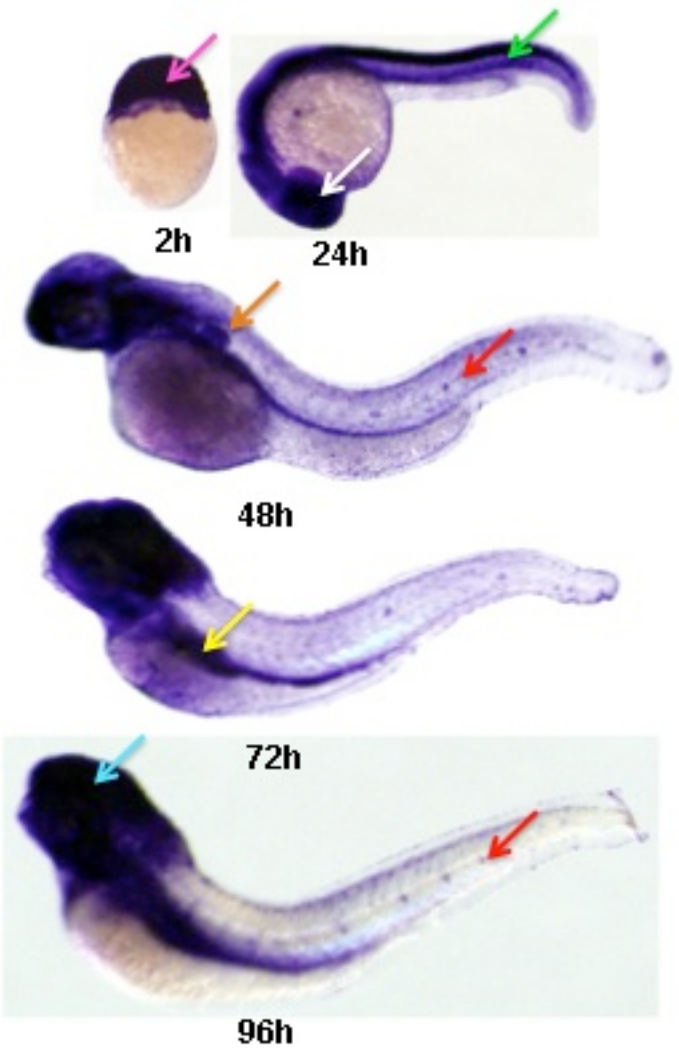


Figure 2.4. Expression pattern of *dnmt6* during embryonic development.

Zebrafish *dnmt6* is a maternally supplied transcript (pink arrow), and is expressed in the eyes (white arrow), brain (light blue arrow), fins (brown arrow), and neural crest cells (red arrow), and gut (yellow arrow).

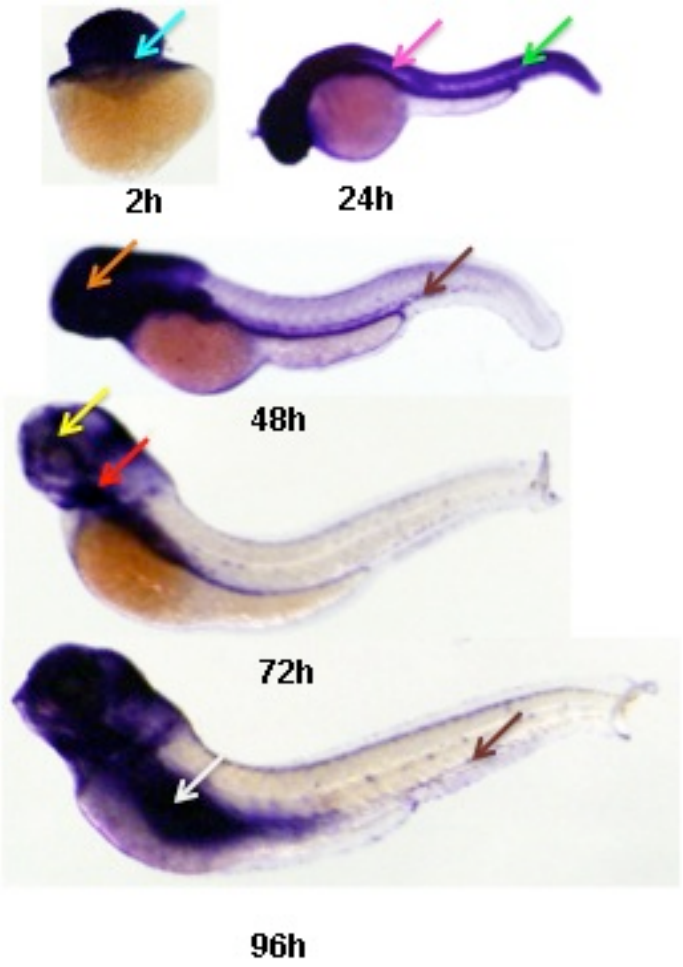


Figure 2.5. Expression pattern of *dnmt7* during embryonic development.

Zebrafish *dnmt7* is a maternally supplied transcript (light blue arrow). *dnmt7* has ubiquitous expression at 72h (pink arrow) suggesting that it may be required extensively during early embryonic development. It is expressed in the eyes (yellow arrow), neural tissue (brown arrow), musculature (green arrow), pharyngeal endoderm (red arrow), gut (white arrow) and a subpopulation of blood cells (brown arrow).

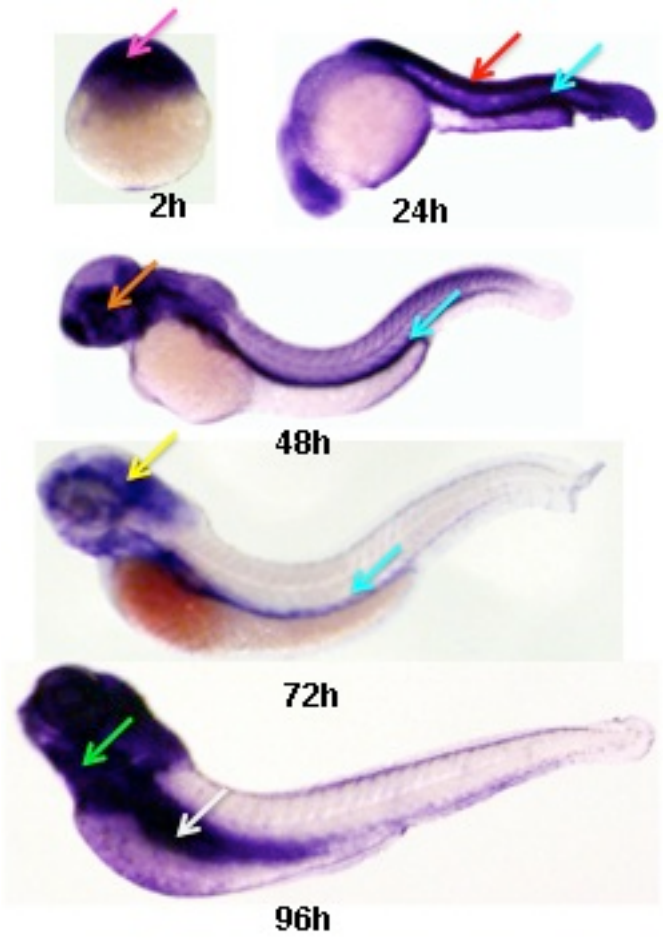
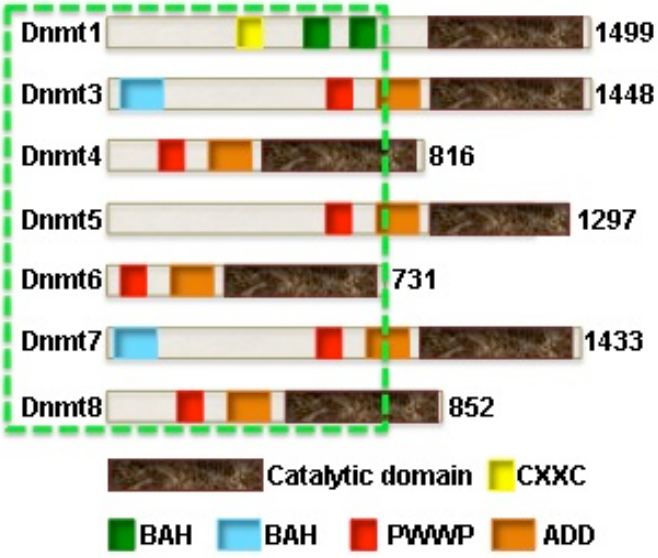
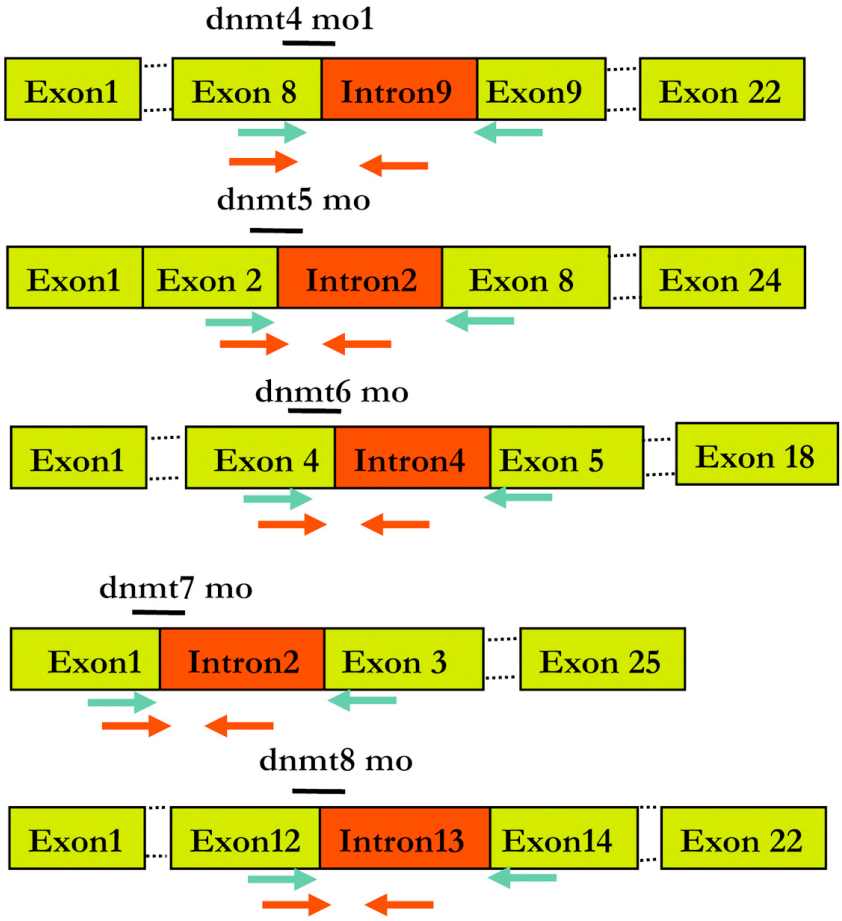


Figure 2.6. Expression pattern of *dnmt8* during embryonic development.

Zebrafish *dnmt8* is a maternally supplied transcript (pink arrow) and is robustly expressed in early development. The expression is prominent in the neural tissue at 24h (red arrow), robustly expressed in the pronephric duct (light blue arrow) at 24h, 48 and 72h. It is expressed in the eyes (orange arrow), brain (yellow arrow), pharyngeal endoderm (green arrow) and the gut (white arrow).

Figure 2.7. Morpholino design. The upper panel represents the domain structure of *dnmts* and the lower panel shows the design of the morpholinos. The green bracket represents the N-termini of the proteins.


Dnmt1
1499
Dnmt3
1448
Dnmt4
816
Dnmt5
1297
Dnmt6
731
Dnmt7
1433
Dnmt8
852
Catalytic domain
CXXC
BAH
BAH
PWWP
ADD


dnmt4 mo1
Exon1
Exon 8
Intron9
Exon9
Exon 22
dnmt5 mo
Exon1
Exon 2
Intron2
Exon 8
Exon 24
dnmt6 mo
Exon1
Exon 4
Intron4
Exon 5
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Exon12
Intron13
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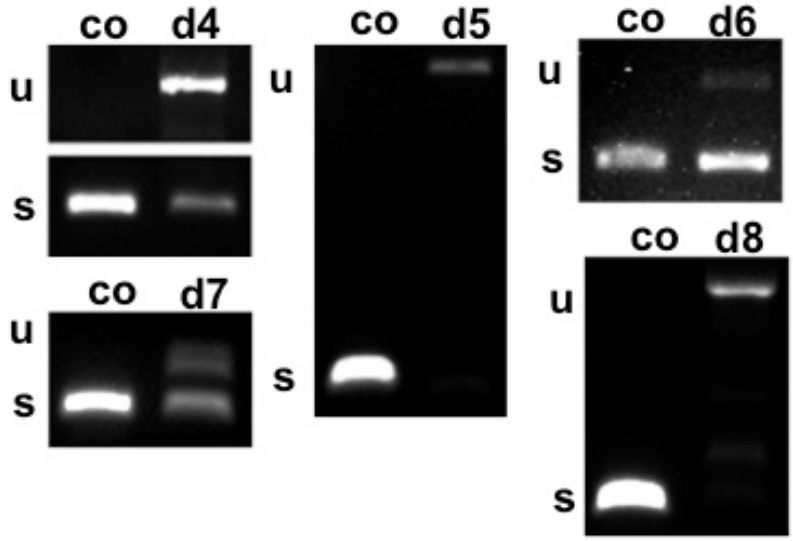

Figure 2.8. Knock down verification of *dnmts* in zebrafish embryos. Transcript levels of *dnmt4, dnmt5*, *dnmt6, dnmt7*, and *dnmt8* were knocked down using antisense morpholinos. Total RNA was harvested from 24hpf controls and morphants. DNase1 treatment was performed before making cDNA. In each PCR reaction a loading control (28S) and water as negative control were used (data not shown). U-unspliced product, s-spliced product, co-control morphant, d4-d8 are dnmt1-8 morphants.

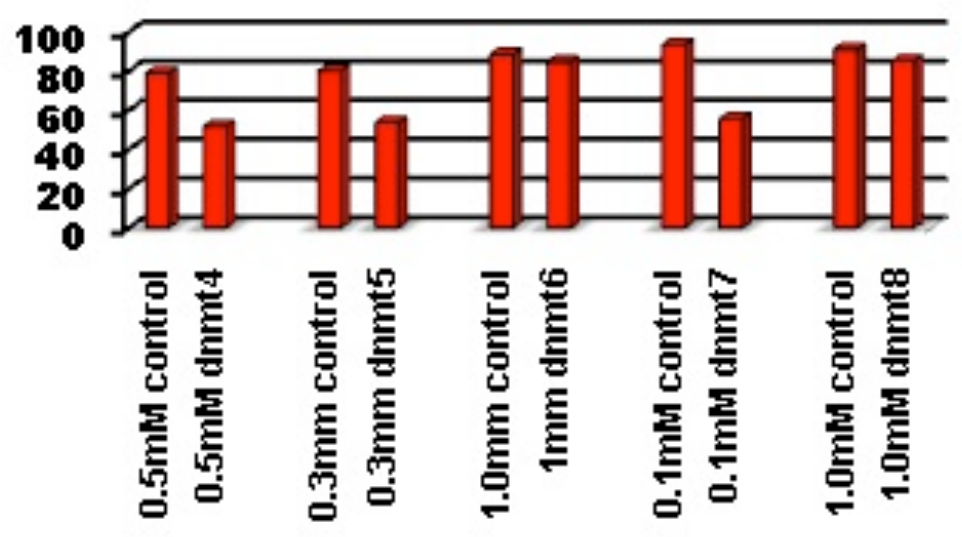

Figure 2.9. Percentage viability of *dnmt* morphants.1-4 cell stage embryos were injected with control or *dnmt* morpholino. The graph is representative of three sets of control or *dnmt* injections (n=3). The pooled total number of injected embryos for each category in the plot is N. N represents total # of embryos injected with *dnmt* morpholino, total # of embryos injected with same concentration of control morpholino. N for *dnmt4*=165,43; *dnmt5*=140/56; *dnmt6*=62,75; *dnmt7*=165,40, and *dnmt8*=97,53. The embryos that survive the *dnmt* morpholino injections displayed characteristic phenotypes of morphants and were embryonic lethal between 72h and 96h. The concentration for each morpholino was determined after titrating four different concentrations.

Figure 2.10. Dnmt morphants gross phenotypes. Gross morphological phenotypes on knockdown of dnmt4, dnmt5, dnmt6, dnmt7, and dnmt8 at 72h**.** Transcript levels of *dnmt4, dnmt5*, *dnmt6, dnmt7*, and *dnmt8* were knocked down using antisense morpholinos. Interestingly, *dnmt4, dnmt5*, *dnmt6, dnmt7*, and *dnmt8* displayed different gross morphological defects suggesting that these enzymes may have different contributions during embryonic development. However, the molecular defects of these morphants have not been characterized.

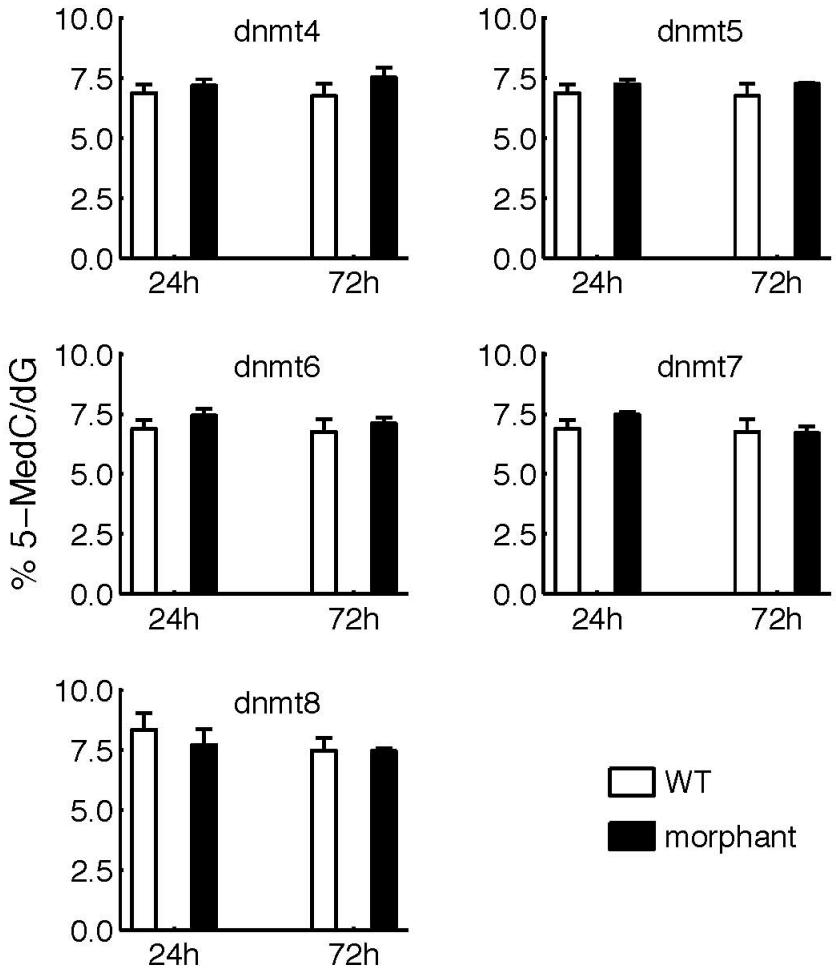

Figure 2.11. Bulk methylation levels in the genomes in *dnmt* morphants. LC-MS quantification was performed to analyze the bulk %5mdC/dG content of dnmt4, dnmt5, dnmt6, dnmt7, and dnmt8 at 24h and 72h. The Y-axis represent the % 5-mdC/dG, the open bars represent the WT and the solid bars represent the morphants. As expected these DNA methyltransferases do not regulate bulk methylation levels. However, it is not known if these enzymes have functions in regulating genes in tissue-specific manner. The data are representative of three biological replicates.

Table 2.1. Morpholino and primer sequences.

Gene	Splice Junction	Morpholino Sequence
dnmt4	e9i8	AGCCACCTGAGGACACACAGGAAGC
dnmt4	e7i8	AGGCATTGTATTTTAGTTACCTCTG
dnmt5	e2i2	TGTCTTTTACCCCTTACCTAGCATC
dnmt6	e5i4	TGGCTACCTGTAAGACAAGACAGTC
dnmt7	e3i2	TGCACCTAAAAACAAGACAAAACAG
dnmt8	e12i13	ACATAATCTGAACTTACAAAGTCCT

Primers.

Gene	Forward Primer-1	Reverse Primer-1
dnmt4-1		
dnmt4-2	GATAATAAGGGCTTTGGTATTGG	CTCAAGGGCCTGGAAGATC
dnmt5	ACACACTGACAACATCAAGAG	tcagttcctcaacagcctat
dnmt6	ATTCAGGTGGATCCAGGC	CTGTTGCCTTTGAGTTCCTTC
dnmt7	cattttattctgttgtctacatattgag	CTCTGTCACTTCTAAATCCC
dnmt8	GTGTTACTGTGTGGAGTGTGTGG	CTGTAGCAATGCCATCAAATAAAGACAG
	Reverse Primer-2	**Forward Primer-2**
dnmt4-1		
dnmt4-2	Cgtgattctgattggctgatg	
dnmt5	TGTCCAAATCCGACACCG	
dnmt 6		ATGGCTACAAATGTTAGTCTGG
dnmt7		ATGGCTACAAATGTTAGTCTGG
dnmt8	Cattcttgctgtgaggcaacagtg	

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CHAPTER 3

DNA METHYLTRANSFERASE 4 REGULATES ZEBRAFISH HEMATOPOIEIS

3.1. Introduction

DNA methylation is an epigenetic mechanism important for transposon silencing, genome defense, imprinting, and gene regulation. During development, DNA methylation affects tissue specific terminal differentiation of various cell types across different vertebrate model systems (1-6). Based on substrate specificity DNMT1 is the primary maintenance DNA methyltransferase and DNMT3a and DNMT3b are the main *de novo* DNA methyltransferases (7), though some crossover likely occurs. Complete loss of *Dnmt1* (8), *Dnmt3a*, or *Dnmt3b* `(9) (separately) are each incompatible with life, thus underscoring the critical role of these enzymes in regulating vital biological processes.

The hematopoietic system is a robust model system to study epigenetic decisions that regulate cell fate commitments (10-12), and aspects of DNA methylation have been addressed previously, primarily in mice. Functional knockout of *Dnmt1* by removal of the catalytic domain of DNMT1 in the HSCs is incompatible with survival (13). On combining the hypomorphic *Dnmt1* allele with the *Dnmt1* null allele, these $Dnmt1^{-/chip}$ mice showed a severe reduction in the

Lymphoid Primed Multipotential Progenitors and a 2.7 fold enrichment of the long-term HSCs. Interestingly, the *Dnmt1*$^{-/chip}$ mice do not exhibit changes in the frequencies of differentiated myeloid and erythroid cells. In contrast, ablation of *Dnmt1* in the hematopoietic compartment obtained by crossing *Dnmt1*$^{fl/fl}$ with interferon-inducible *Mx-Cre* transgenic mice do not exhibit hematopoietic defects in terminally differentiated blood and progenitor cell type (14). This observation suggests that although *Dnmt1* may not be required for terminal differentiation of different lineages in hematopoiesis, roles for *Dnmt3a* and/or *Dnmt3b* remain. However, in transplantation assays (14) *Dnmt1* was shown to be required for self-renewal and differentiation of the HSC cells and for differentiation into mature myeloid cells. The studies discussed above do not reconcile, and indicate that *Dnmt1* is either required solely for the generation of terminally differentiated cells of the lymphoid lineage (13) or the myeloid lineage (14). Also, this suggests that *Dnmt3* family genes may play a role in regulating formation of terminally differentiated hematopoietic cells.

Knock out of both the *de novo* DNA methyltransferases *Dnmt3a* and *Dnmt3b* in HSCs focused on the requirement of *de novo* methylation in HSC self-renewal (15). However, knock out of both *Dnmt3a* and *Dnmt3b* in HSCs did not confer defects in the generation of lineage specific progenitors. This observation strongly suggests that *de novo* methylation is dispensable for lineage commitment process. However, neither this nor other studies (10, 13, 14) have investigated the contributions of *Dnmt3a* and *Dnmt3b* in terminal differentiation of mature blood lineages, which is the focus of our study.

In this report we show that zebrafish *dnmt4* a homolog of the human *DNMT3B* regulates terminal differentiation of erythroid and myeloid lineages. *dnmt4* morphants show a downregulation of erythroid and myeloid lineage cells. However, immature cells of both these lineages are expressed robustly suggesting that anemia and neutropenia occur due to defects in terminal differentiation rather than due to defects in the formation of lineage precursor cells. Notably *dnmt4* morphants also display cranio-facial anomalies that together with the blood development abnormalities are reminiscent of the features observed in ICF (***I***mmunodeficiency ***C***ranio-***F***acial anomalies) syndrome arising due to mutations in *DNMT3B*. Because *dnmt4* morphant embryos exhibit molecular defects that largely mimic defects in the ICF syndrome these morphants might be used as an *in vivo* system to dissect molecular pathways associated with this disease. We further show that retinoic acid signaling is perturbed in *dnmt4* morphants and exogenous supplementation of ATRAL rescues the neutrophil lineage defects in *dnmt4* morphants. Moreover, retinoic acid deficiency created by both genetic knockdown and pharmacologic inhibition of the pathway phenocopy the defects observed in *dnmt4* morphants.

3.2. Materials and methods

3.2.1. Zebrafish maintenance

Zebrafish stocks were maintained in z-mods on a 14:10 hour light:dark cycle at 28°C using standard fish husbandry IACUC protocols. Transgenic Tg(*fli1a:*GFP) zebrafish were obtained from the N. Trede Laboratory.

3.2.2. Zebrafish microinjections

Morpholino oligonucleotides were ordered from Gene Tools LLC. The sequences for *dnmt 4*, *5*, *6*, *7*, *8*, *p53*, *rdh1l* and control morpholinos are provided in Chapter 2 and Table 3.1. The morpholino sequences for *dnmt1* (4) and *dnmt3* (2) have been described elsewhere. Human *DNMT3B*, and *DNMT3B* catalytic mutant plasmids have been described elsewhere (2). Human *DNMT3A* plasmid was purchased from Origene. For RNA injections, 12pg of human *DNMT3A*, 12pg of human *DNMT3B* and catalytically inactive human *DNMT3B* plasmids and 15pg of capped and polyadenylated zebrafish *dnmt4* mRNA were injected into one-cell stage wild type zebrafish embryos to test for rescue the defects in *dnmt4* morphants.

3.2.3. Oligonucleotides and constructs

The GenBank accession numbers for *dnmt4*, *5*, *6*, *7*, and *8* are NM_001025450, NM_001020479, NM_001018140, NM_001020476 and NM_001018134 respectively. Zebrafish *dnmt4*, *5*, *6*, *7* and *8* were cloned in pCRTOPOII vector. MJR provided clones for *mpx*, *cmyb*, *spi1*, *gata1*, and SAH provided clones for *alphae1*, *alphae2*, *betae1*, *betae3*. The oligo nucleotide sequences for cloning and confirming the splice check of the DNA methyltransferases have been provided in and 3.1.

3.2.4. Whole mount *in situ* hybridizations

Whole mount *in situ* hybridizations were carried out as described previously (16) using digoxigenin-labeled riboprobes for *dnmt4*, *dnmt5*, *dnmt6*, *dnmt7*, *dnmt8*, *mpx*, *cmyb*, *spi1*, *gata1*, *alphae1*, *alphae2*, *betae1*, *betae3,* *ascl1a*, and *ifabp*.

3.2.5. Alcian Blue Staining

Alcian blue staining has been described in detail previously (2). The stained embryos imaged with Zeiss Axiovert100 microscope using an Olympus Magnafire color camera.

3.2.6. Histological analysis

In situ stained embryos at mentioned time points were fixed, processed, and sectioned for histological analyses as described previously (2). The sections were photographed with Zeiss Axiovert100 microscope using an Olympus Magnafire color camera.

3.2.7. Whole mount double fluorescent *in situ* hybridizations

Digoxigenin (DIG)- and fluorescein (FITC)-labeled UTPs were used to *in vitro* transcribe *dnmt4* and *cmyb* antisense probes according to the manufacturer's instructions (Roche, Palo Alto, CA). Embryos were fixed and processed as described for WISH. Cy3:Tryamide and FITC:Tryamide were synthesized following protocols mentioned on the website Xenbase. *Dnmt4*-Dig

probe was incubated with primary antibody (anti-DIG-POD Fab Fragments 1:1000 dilution in block) and developed in PBST, 0.01 M Imidazole and 1:500 tyramide–Cy3. *Cmyb*-Fluo probes were detected with anti-Flu-POD Fab Fragments 1:1000 and developed in PBSTI, 0.001% H2O2 and 1:1000 tyramide-FITC at room temp for 60 min. The embryos were photographed using Olympus FV1000XY microscope and imaged with Fluoview software.

3.2.8. Histochemical staining

Hb activity was detected in whole embryos by histochemical staining for Hb using o-dianisidine as described previously (17). Sudan Black histochemical staining for detecting lipophilic mature neutrophils was performed as described previously (18) . The embryos were stored in 80% glycerol and photographed with Zeiss Axiovert100 microscope using an Olympus Magnafire color camera.

3.2.9. Morphometry

To analyze the morphology of the circulating peripheral blood cells tails of 72h embryos morphometric analyses was performed as described (19). The cells were imaged at high power using Zeiss Axiovert100 microscope using an Olympus Magnafire color camera.

3.2.10. Genomic DNA isolation and global DNA methylation assay by liquid chromatography-mass spectrometry

Embryos were collected at 24 and 72 hpf, and genomic DNA was isolated using Gentra Systems PURGENE DNA Purification kit. Global DNA methylation analysis was performed using liquid chromatography-mass spectrometry as described previously (20).

3.2.11. PCR, gene expression and methylation analysis

Total RNA was harvested and treated with DNase1 to remove gDNA contamination. For whole genome expression analyses 24h and 72h RNA from WT and *dnmt4* mo1 were labeled with Cy3 and Cy5 dyes and hybridized on Agilent 2-color Zebrafish v3 Gene Expression (4x44K) microarray in triplicates. cDNA for PCR reactions was synthesized from 2 µg of total RNA using Superscript III (Invitrogen). PCR was performed using the Roche Lightcycler instrument and software, version 3.5 (Roche Diagnostics). A template-free negative control was included in each experiment. The sequences of PCR primers used have been provided in Table 3.1.

3.2.12. MeDIP (Methylated DNA Immuno Precipitation) and array hybridization

The procedure used for MeDIP has been described previously (21). The immuno precipitated DNA was amplified using whole genome amplification kit

(Sigma Aldrich). The amplified DNA was labeled with Cy5 and Cy3 and was competitively hybridized to custom-designed 244K oligonucleotide zebrafish promoter arrays (Agilent Inc).

3.2.13. Statistical analyses for whole genome expression and MeDIP analyses

The probes on the tiling arrays were mapped to the most recent zebrafish genome (Zv9). Statistical analysis of the MeDIP-chip datasets was performed using the SLAM software (http://jlab.byu.edu/SLAM/), which is adapted from normalization method described previously (22). The gene expression data were also normalized using a variation Song et al (22). GoMiner (23) was used to identify under and over represented Gene Ontology terms.

3.2.14. Statistical analyses

Prism 4 (GraphPad Software, San Diego, CA) was used for statistical analyses. Results of statistical analyses are provided in Table 3.2. Values were calculated using Student's t test. The quantitative data is representative of at least three independent experiments.

3.2.15. Drug treatments

To rescue the retinoic acid deficiency in *dnmt4* and control morphant embryos were treated with 0.3μM all-*trans*-retinaldehyde (RAL; Sigma-Aldrich) at 10h, 24h, 48h and 72h for 30 min. After each treatment the embryos were

washed three times with embryo water. For creating retinoic acid deficient embryos DMSO or 5μM 4-diethylamino benzaldehyde (DEAB; Sigma-Aldrich) a competitive inhibitor of aldehyde dehydrogenases was added to plates containing wild type embryos at 5h. The embryos were allowed to grow till 72h.

3.3. Results

3.3.1. *dnmt4* is expressed in the hematopoietic cells in zebrafish larvae

To clarify the roles of DNA methylation during various steps of hematopoiesis we asked which of the DNA methyltransferases are expressed in the hematopoietic compartment of the zebrafish embryos. Unlike mammals, zebrafish have eight DNA methyltransferase genes. Based on sequence homology and functional complementation experiments *dnmt1* closely resembles human *DNMT1 (4)*. Zebrafish *dnmt2*, like human *DNMT2,* behaves functionally as an RNA methyltransferase (24), and therefore was not addressed further. There are six *DNMT3*-family genes in zebrafish in comparison to the three mammalian genes (25). Based on the sequence alignments *dnmt3, dnmt4, dnmt5 and dnmt7* resemble *DNMT3B*, where as *dnmt6 and dnmt8* demonstrate higher sequence similarity to *DNMT3A.* However, complementation and/or biochemical analyses with human proteins are required to determine the functional homology of the zebrafish proteins to their human counterparts. To determine which of the *dnmt*s are expressed in the hematopoietic compartment of the zebrafish embryos we performed *in situ* hybridization analyses of the

zebrafish *dnmt1, dnmt3, dnmt4, dnmt5, dnmt6, dnmt7 and dnmt8.* We also cross sectioned the embryos at 72h to determine the anatomical location of the different transcripts. Interestingly, we find that *dnmt4* is a maternally-supplied transcript is robustly expressed in the hematopoietic compartment of zebrafish embryos at 24h, 48h, 72h, and 96h (Figure 3.1). However, it is not limited to the blood lineage. The expression of the *dnmt4* transcript is ubiquitous in the anterior structures, including parts of the brain, eyes, pharyngeal arches skeleton, and pectoral fin musculature. By 72h the expression of *dnmt4* becomes very specific and is robustly expressed only in the tegmentum, telencepalon, diencephalon, ventricular zone, retina, parynx, gut, pectoral fin, pharyngeal arches, artery and hematopoietic cells (Figure 3.1). Notably, in the posterior structures *dnmt4* expression is limited to the hematopoietic compartment such as the artery and a subset of the blood cells. *dnmt4* sense strand probe was used as a control (Figure 3.1) and showed no signal.

We next analyzed the anatomical locations of the antisense *dnmt4* probe by cross-sectioning digoxigenin labeled antisense *dnmt4* probe stained embryos at 24h, 48h, and 72h. (Figure 3.2) shows the expression of *dnmt4* in the dorsal aorta of the 72h embryo. Cross-sections of 24h and 48h *dnmt4* stained embryos also show robust expression of *dnmt4* in the dorsal aorta (data not shown). To further test for *dnmt4* expression in the hematopoietic cells of the zebrafish embryos we performed double fluorescent *in situ* hybridization analysis of fluorescein labeled *cmyb* antisense probe and cy3 labeled *dnmt4* antisense probe (Figure 3.2 lower panel). *dnmt4* transcripts co-localize with the transcripts

of *cmyb* in the hematopoietic cells present in the caudal hematopoietic tissue (CHT) of the embryos at 72h. This data is consistent with a previous report (10) that shows strong expression of murine *Dnmt3b* in the hematopoietic stem cells and uncommitted progenitor cells.

We next addressed the expression of all other DNMTs in the hematopoietic cells at 72h (Figure 3.3). In spite of a significant overlap in the expression patterns of these genes in various organ systems the other DNA methyltransferases *dnmt1, 3, 5, 6, 7, and 8* are not expressed in the hematopoietic compartment (by *in situ* hybridization analyses), the CHT region of the zebrafish embryos at 72h. We cross sectioned *in* situ stained embryos to investigate the anatomical localization of these transcripts. Although *dnmt4* is expressed in other tissues, those tissues also harbor other DNMT3 paralogs. In contrast, the apparent specificity of dnmt4 for expression in the blood lineage at 72h prompted our examination of roles for dnmt4 in blood development, as the potential for redundancy with other DNMTs is minimized.

3.3.2. Transient knockdown of *dnmt4* with antisense morpholino

We tested a role for *dnmt4 in* zebrafish hematopoiesis by a loss-of-function approach using antisense morpholinos. The domain structure of dnmt4 has a characteristic regulatory N terminal with the PWWP and PHD-like domains and an evolutionary conserved C terminal catalytic domain (Figure 3.4). Due to the lack of availability of antibodies against the zebrafish Dnmt proteins we have

used morpholinos designed to interfere with RNA splicing instead of those blocking translation. We designed two nonoverlapping antisense morpholinos, one against the exon 8 and intron 9 splice junction of *dnmt4* (mo1, as shown in Chapter2) and a second against the exon 7 and intron 8 junction (mo2, data not shown). We also used a control morpholino bearing a five base pair mismatch from the first morpholino design.

We find that *dnmt4* mo1, but not the control morpholino, blocked the formation of the spliced product with an efficiency of nearly 90% (Chapter 2, Figures 2.7 and 2.8). On sequencing the unspliced product we found that the retention of the intron 9 induced a stop codon in the protein before the PHD-like domain (indicated by an asterisks in Figure 3.4). This suggests that the *dnmt4* transcript containing the unspliced intron will not lead to the formation of a functional protein.

We have previously demonstrated that *dnmt1* is a maintenance DNA methyltransferase in zebrafish as antisense morpholino knockdown of *dnmt1* but not *dnmt3* lead to a reduction in global methylation levels (2,4). Consistent with reports that *Dnmt3b* family enzymes are *de novo* DNA methyltransferases and their knockout does not lead to decrease in global DNA methylation levels (9), we observed no significant changes in the levels of global DNA methylation (as measured by LC-MS) in *dnmt4* morphants in comparison to the wild type embryos at 24h and 72h (Chapter 2, Figure 2.11) . Also, in agreement with this idea that zebrafish *dnmt4, 5, 6, 7,*and *8* are homologs of human *DNMT3A* or *DNMT3b* and that they are not required in maintenance of bulk methylation

levels, antisense splice blocker morpholino knockdowns of *dnmt5*, *6*, *7*, and *8* also do not lead to a loss in genome-wide methylation of the zebrafish embryos as measured by LC- MS quantification (Chapter 2, Figure 2.11). To test whether the defects in the *dnmt4* morphant embryos were specific, we tested *dnmt4* mo2, which conferred similar phenotypic and molecular defects (data not shown). This stark similarity produced by the two morpholinos and the rescues by *dnmt4* RNA (Figure 3.8.) help establish the specificity of the *dnmt4* knockdown.

3.3.3. *dnmt4* morphants have specific defects

Transient knock down of *dnmt4* by both the morpholinos mo1 and mo2 cause lethality between 3 and 4dpf, underscoring the necessity of this enzyme for viability. Around 24hpf the yolk sac extension started to become thinner and by 48hpf the embryos lost their yolk sac extension. The prominent distinguishing phenotypes of the dnmt4 morphants at 72hpf were lack of pectoral fins, hydrocephalus of the hindbrain, defects in formation of jaws, smaller eyes and head. The body of the morphant was thinner with tail curvature and displayed a lack of yolk sac extension. Also, these morphants showed pericardial edema and hypochromic blood cells circulating in the vasculature. These defects were not observed in the *dnmt4* control morpholino injected embryos (Figure 3.4). To determine if the *dnmt4* morphants were developmentally delayed and were defective in the formation of multiple organ systems we analyzed them for the formation of the gut and the brain. We chose these organs because *dnmt4* is robustly expressed in these structures. Also, we have earlier established that

dnmt1 and *dnmt3* in zebrafish are required for the terminal differentiation of intestinal cells and brain, respectively (2,4). In support of the idea of tissue-specific functions of the DNA methyltransferases we show that d*nmt4* morphants do not exhibit a terminal differentiation defects in the intestines and brain as seen by the expression of *ifabp* and *zash1a* respectively (data not shown). Apart from the expression of *dnmt4* in the hematopoietic compartment *dnmt4* is also robustly expressed in the immature eyes as seen in the cross section of 24hpf embryos stained with *dnmt4* (data not shown). Importantly, knockdown of *dnmt4* causes disruption of the entire eye morphogenesis. The *dnmt4* morphant eyes are mirophthalmic, have abnormal lens structure and exhibit a complete lack of retinal lamination. At the molecular level *dnmt4* morphants harbor proliferating retinal progenitors (*crx)*, amacrine cells, immature cone and rod cells (*neurod)*, but show reduced differentiated cells of retinal ganglion cell layer and retinal nuclear cell layer (*isl1).* Moreover *dnmt4*, morphants lack expression of *irbp* (interphoto receptor retinoid binding protein), a marker for terminally differentiated retinal cells. Interestingly the overall developmental defects in the gross morphology of the *dnmt4* morphants recapitulate the defects seen in the murine *Dnmt3b* knockout model and are also reminiscent of some of the defects observed in ICF syndrome arising from missense mutations in DNMT3B (26). Although we find other affected tissues, this study focuses on the impact of *dnmt4* on blood development.

3.3.4. Erythrocyte maturation is affected in *dnmt4* morphants

Because *dnmt4* was found to be expressed in the hematopoietic compartment of zebrafish embryos we wanted to know if the *dnmt4* morphants were compromised in the formation of blood cells. Indeed the *dnmt4* morphants were severely anemic and showed a lack of hemoglobinization as measured by reduction in o-dianisidine positive cells at 48hpf and 72hpf (Figure 3.5). However, there was no apparent defect in specification of the red blood cells as observed by the expression of *gata1*, *alphae1, alphae2* and *betae1* globin expression (Figure 3.6). To determine the morphology of erythrocytes in *dnmt4* morphants we performed them by Wright-Giemsa staining. Erythrocytes in 72hpf *dnmt4* morphant embryos appeared immature in comparison to the control morphants (Figure 3.5). The morphant blood cells appeared to have a decondensed nuclei with an increased nuclear to cytoplasmic ratio. Notably the cytoplasm of the *dnmt4* morphant erythrocytes showed a purplish staining of the cytoplasm instead of the reddish hue observed in hemoglobinized control cells. To determine whether this lack of hemoglobinization of the peripheral blood cells in the morphant embryos was due to the morpholino-induced increased levels of *p53 (27)* we co injected the *dnmt4* morpholino with a *p53* morpholino and observed that this did not rescue the robust downgulation of o-dianisidine stained cells (data not shown). This lack of rescue suggested that defects in erythroid

maturation in the *dnmt4* morphants was not due to off-targets effects of the morpholino.

3.3.5. *dnmt4* morphants are neutropenic

To determine if *dnmt4* morphants harbored defects in other hematopoietic lineages we interrogated expression of myeloid lineage markers. Interestingly *dnmt4* morphant embryos showed robust down-regulation of terminal neutrophil differentiation as tested by staining with the lipophilic dye Sudan Black (Figure 3.7). Similar to the erythroid lineage, progenitors of the neutrophil lineage and immature neutrophils persisted in morphants as evidenced by the expression of *spi1* (data not shown) and *mpo* respectively (Figure 3.7). Interestingly the *mpo* positive cells were also found to be mislocalized outside the CHT region. This could be a result of genes and pathways required for homing of the neutrophils. This observation further corroborates the previous reports that myeloid-specific genes *Mpo*, *Cxxcr* and *Gadd45a* have differential levels of methylation in uncommitted and committed progenitor cells (10). We were unable to investigate the effects of *dnmt4* knockdown on T and B cell development because of morphant lethality (around 3.5 days) prior to lymphoid population of the thymus (28).

3.3.6. *dnmt4* morphants do not have vasculature defects

Since hematopoietic stem cells and the endothelial cells in the aorta gonad mesonephros region are believed to arise from common precursor cells of

hemogenic endothelium (29) we asked if similarly to blood cells, formation of the vasculature was also defective in the *dnmt4* morphants. To this effect we injected *dnmt4* and the control morpholinos into the Tg(*fli1a:*GFP) zebrafish. Confocal imaging of transgenic morphant embryos revealed that *dnmt4* morphants had intact vasculature (data not shown). This again suggested that rather than pervasive developmental defects, *dnmt4* morphants harbored tissue-specific defects. *dnmt4* morphants showed defects in terminal differentiation of the hematopoietic cells but the vasculature formation was unaffected. We attributed the distortion of the inter-somitic vessels from V- to U shape, to the malformation of the somites rather than to defects in vasculature formation itself. This observation is in agreement with a previous report that treatment of zebrafish embryos with the demethylating agents 5-azacytidine and 5-aza-2deoxycytidine leads to abnormal somite patterning (30). However, the exact contribution of DNA methyltransferases in somite formation and patterning requires further investigation.

3.3.7. Human *DNMT3B* complements *dnmt4* loss in zebrafish

To address the functional relevance of our findings, we sought to determine in *DNMT3B*, the human homolog of *dnmt4* could rescue *dnmt4* morphants. Toward this goal, we co-injected GFP-tagged clones of catalytically active and inactive versions of human DNMT3B with *dnmt4* morpholino (Figure 3.8). Indeed, as hypothesized, catalytically active human DNMTB but not the

inactive derivative rescues *dnmt4* morphants. This suggests that zebrafish *dnmt4* and human *DNMT3B* are functionally homologous and that catalytic activity of the DNMT protein is required for its function in hematopoietic cells. The co-injection of human DNMT3B partially rescued the defects of the erythroid lineage as seen by increase in the o-dianisidine positive cells (Figure 3.9) and myeloid lineage evidenced by the rescue of mislocalized of *mpo*-positive cells (Figure 3.9). In addition to the partial rescue of hematopoietic differentiation, catalytically active DNMT3B also markedly improved gross morphological defects in *dnmt4* morphants (Figure 3.8). Recent reports (31,32) have shown that mutations in DNMT3A are involved in MDS and AML. To determine if *dnmt4* was functionally similar to human DNMT3A we tried to rescue *dnmt4* morphants with human DNMT3A. Co-injection of GFP-tagged plasmid containing full length human DNMT3A did not rescue the defects of *dnmt4* morphants (data not shown). To verify that co-injected DNMT3A was expressed *in vivo* in *dnmt4* we over-expressed GFP-tagged DNMT3A constructs by transient transfection in SW480 cells. As expected we observed expression of GFP in the nucleus of the cells suggesting that these fusion proteins were indeed being expressed and were localizing to the nucleus correctly (Figure 3.8).

3.3.8. Hematopoietic defects are not recapitulated in other *dnmt* morphants

We hypothesized that *dnmt4* regulates zebrafish hematopoiesis in a tissue-specific manner. To show that this defect was specific to *dnmt4* we

knocked down the levels of the other DNA methyltransferases *dnmt1*, *3*, *5*, *6*, *7* and *8*. The design of the morpholinos and the verification of the knockdown of transcripts of *dnmt5*, *6*, *7* and *8* are shown in Chapter 2, Figures 2.7 and 2.8). The comparison of the 72hpf morphant phenotypes of the *dnmt4, 5*, *6*, *7* and *8* show different morphological phenotypes resulting form transient knock down of these genes. However, *dnmt5*, *6*, *7* and *8* morphants do not show mis-expression of o-dianisdine (Figure 3.12) *mpo*-positive cells (Figure 3.13). Also, *dnmt1* and *dnmt3* morphants at 72hpf do not exhibit hematopoietic defects as shown by the o-dianisidine staining (data not shown) and normal expression of the *mpo*-positive cells (data not shown).

3.3.9. Dnmt4 morphants are retinoic acid deficient

We wanted to investigate the pathways that were disrupted in the *dnmt4* morphants. We decided to take a candidate approach and investigated different pathways that could be downstream targets of *dnmt4*. In this approach we took hints from our 72h whole genome expression array data and overall defects observed in the *dnmt4* morphants. One of the candidate pathways that we investigated was retinoic acid (RA), signaling pathway. This pathway is conserved across species and is required for proper embryonic development of multiple tissues (33). Both excess and deficiency of this morphogen causes teratogenicity in embryos. Retinoic acid deficiency in zebrafish embryos causes pleiotropic effects some of which include loss of pectoral fins, heart enlargement, loss of jaw formation, defects in retina, brain, somite, and intestinal development

(34). Apart from these tissues RA is required for the formation of hematopoietic cells (35). Importantly, RA is successfully used in clinic as differentiation inducing therapy for the treatment of acute promyelocytic leukemia (APL).

3.3.10. Treatment of dnmt4 morphants with retinoic acid rescues *dnmt4* morphants

As our results suggested retinoic acid deficiency in *dnmt4* morphants, we asked if treatment with all-trans retinaldehyde would rescue the mislocalization of myeloid cells in *dnmt4* morphants. All-trans retinaldehyde treatment rescued the mislocalization of the *mpo* positive cells in the *dnmt4* morphant embryos, corroborating our hypothesis that the RA pathway is one of the major downstream targets of *dnmt4* (Figure 3.10).

3.3.11. Retinoic acid deficiency phenocopies *dnmt4* morphants

To evaluate if retinoic acid deficiency in WT fish would lead to similar defects in *mpo*-positive cells and terminal differentiation of the neutrophils we treated wild type embryos with DEAB, a nonspecific inhibitor of retinaldehyde dehydrogenases in zebrafish (Figure 3.11). In accordance with our hypothesis that retinoic acid deficiency causes lack of terminal differentiation of the myeloid population in zebrafish embryos we observed that the DEAB-treated embryos phenocopied both the myeloid and erythroid misregulation of the *dnmt4* morphants (Figure 3.11). To determine if genetic knockdown of RA biosynthetic

pathway would yield similar results we knocked down *rdh1l* in embryos. The *rdh1l* morphants phenocopied both *dnmt4* morphants and DEAB treated embryos (Figure 3.11), supporting a causative role for retinoic acid deficiency in the hematopoietic defects in of *dnmt4* morphants.

3.4. Discussion

In the present study we have identified that zebrafish *dnmt4*, a homolog of human DNMT3B regulates terminal differentiation of erythroid and myeloid cells. Terminal differentiation of blood lineages is an important process for converting immature committed cells to mature functional cells. Defects in this process are often observed in hematological disorders like Myelodysplastic Syndromes (MDS) (36). MDS are disorders of the HSCs that are characterized by ineffective hematopoiesis leading to peripheral blood cytopenias. Also, hematopoietic diseases harbor aberrant expressions of DNMTs (37,38), and mutations of *de novo* DNA methyltransferases (32,39). A striking hallmark of hematopoietic diseases is *de novo* promoter hypermethylation (38,40-46) and subsequent transcriptional silencing of tumor suppressor genes. This phenomenon may be associated with either transcriptional deregulation of DNA methyltransferases themselves or the interacting partners of the DNMTs that may recruit them aberrantly to promoters of regulatory proteins. Thus to better understand deregulations associated with hematopoietic transcriptional programs exact contributions of different DNA methyltransferases warrants further investigations.

The principal findings of this report are that (1) *dnmt4* is expressed in the hematopoietic compartment of the zebrafish. (2) Only *dnmt4* and not the other *dnmts* are required for the formation of the terminally differentiated cells of the myeloid and erythroid lineages. (3) The defects in *dnmt4* morphants are rescued with complementation of the functionally active version of the human *DNMT3B*. (4) The defects in the maturation of the myeloid cells of the *dnmt4* morphants are rescued by treatment of ATRAL. (5) Both pharmacologic and genetic knockdown of the retinoic acid biosynthetic pathway phenocopies the hematopoietic defects in dnmt4 morphants. Our study provides a model where retinoic acid mediates the effect of the *de novo* methyltransferase *dnmt4* during normal blood development in zebrafish.

To model the ICF syndrome mice carrying two missense that lead to partial loss of function of *Dnmt3b* were generated (26). These mice phenocopy some aspects of the human ICF disease. They displayed facial dysmorphism and low weight at birth. However, unlike agammaglobinemia that is observed in most patients these mice display defects in T cell lineage. After birth, the number of thymocytes in ICF mice were reduced due to apoptosis in the thymus suggested that *Dnmt3b* activity is essential for the survival of T cells in the thymus. However, unlike ICF patients these mice did not display changes in mature B cell populations. Interestingly, decreased levels of T cells are not as frequently observed in ICF patients Instead, the patients largely suffer from agammaglobinemia. However, atleast one patient has been described with suffering from anemia and neutropenia (47). These findings suggest that the ICF

patients suffer from multiple complications and the clinical manifestations may be diverse.

At the cytogenetic level the ICF mice display hypomethylation of centromeric minor satellite DNA and juxtacentromeric major satellite DNA. Perhaps, such differences in cytogenetic pathophysiology may arise because of the differences in the genomes of mice and humans. Thus mice show more extensive DNA hypomethylation and the kind of immunological dysfunction is different. Importantly, because of the rare numbers of available ICF patient samples (nearly 50 reported cases), it is be important to develop new model systems that will recapitulate different aspects of the disease. Because most disease models only phenocopy certain aspects of the disease cumulative data from multiple model systems may be useful in generating a better understanding of the disease. Here we present data describing a zebrafish model of hypomorphic *DNMT3B* that partially phenocopy the ICF patients in harboring cranio facial defects of ICF patients. In addition they exhibit profound neutropenia and anemia. The *dnmt4* morphants will be useful in dissecting the functions of DNA methylation in cranio facial development and developmental roles of *DNMT3B* that may be critical for the development of ICF syndrome.

3.5. Acknowledgements

I would like to thank the following for protocols, and/or technical help. N.S.T., M.J.R., for technical help, protocols and suggestions. S.R.J. and A.R.K.

did global methylation analysis on *dnmt* morphants; E.J. did expression and MeDIP microarray analysis. S.C., helped with *dnmt in situ* stained cross sections.

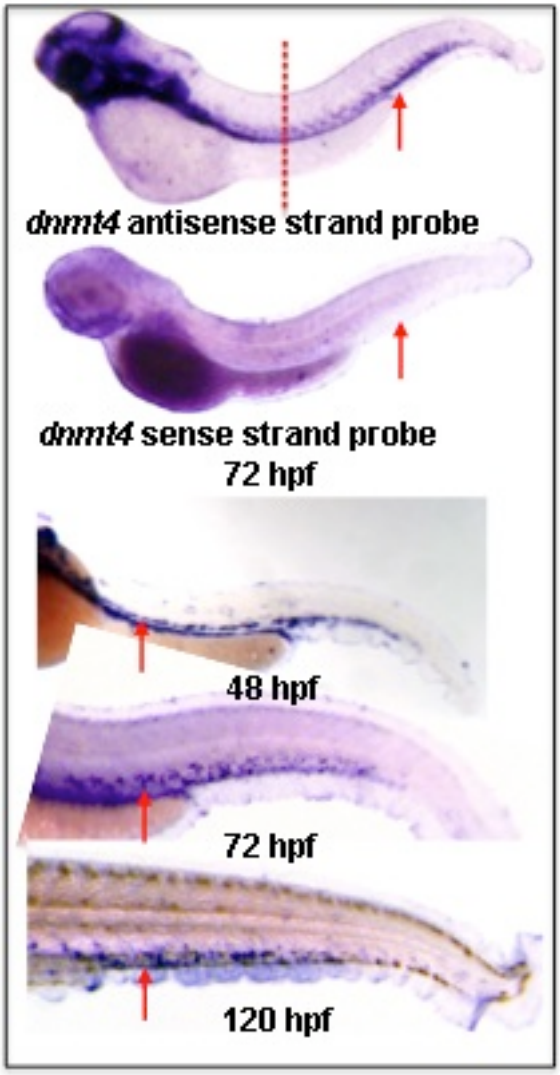


Figure 3.1. *dnmt4* is expressed in the hematopoietic cells in zebrafish. Whole mount in situ staining for *dnmt4* in zebrafish at 72h using antisense strands and 72h sense strand. Comparison of expression pattern of *dnmt4* at 48h, 72h and 120h showing the expression of *dnmt4* in the hematopoietic compartment of the embryos.

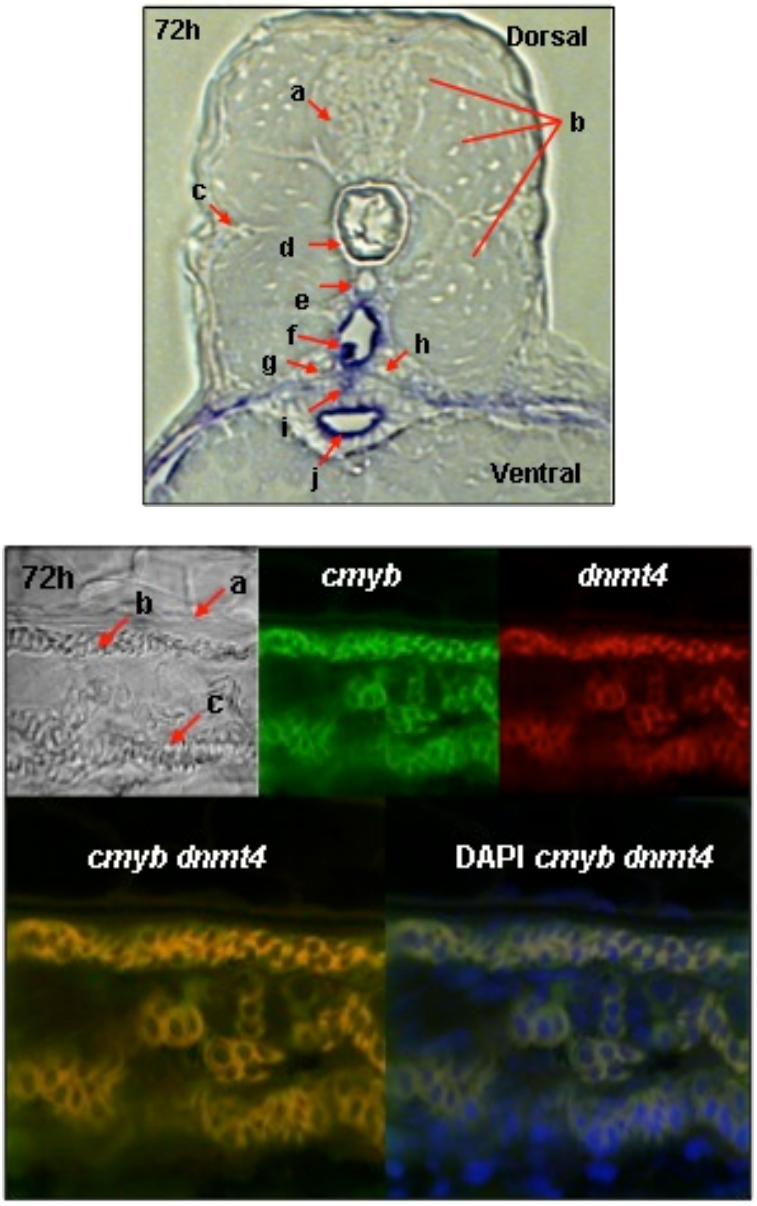


Figure 3.2. *dnmt4* is expressed in the hematopoietic cells. Cross section of *dnmt4* stained fish at 72hpf a-neural tube, b-myotomes, c-horizontal septum, d-notochord, e-hypochord, f-dorsal aorta, g & h –pronephric ducts, i-posterior cardinal vein, j-gut. Lower panel shows *dnmt4* expression co localizes with expression of *cmyb* in the caudal hematopoietic region.

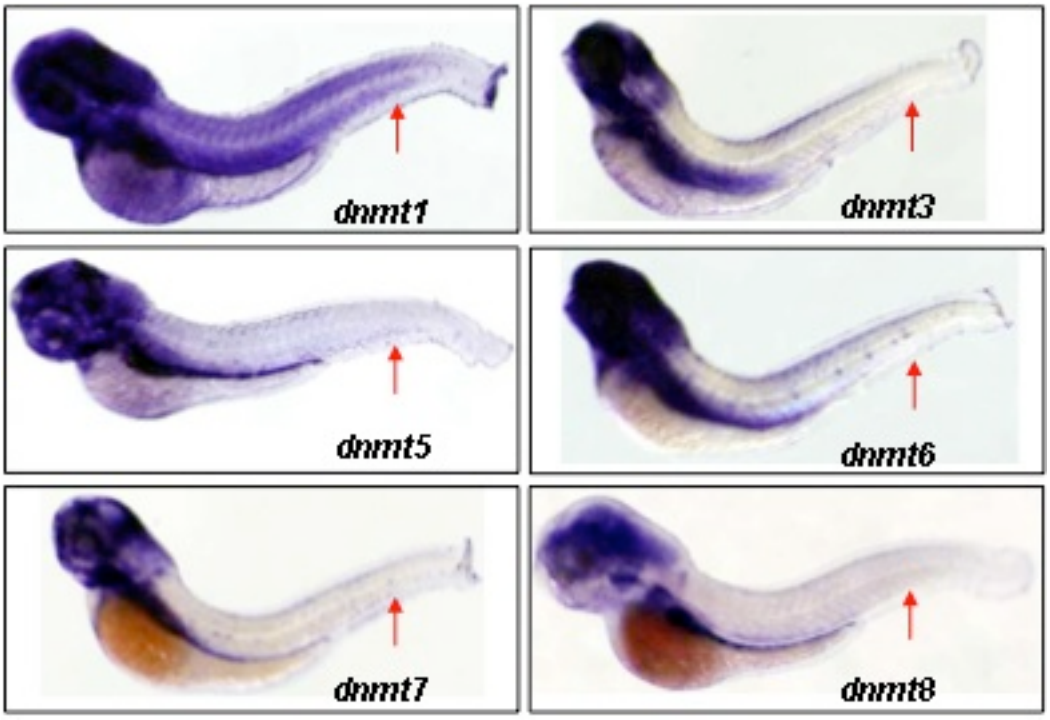


Figure 3.3. Other *dnmts* are not expressed in the hematopoietic cells in zebrafish embryos at 72h. To determine whether there is redundancy in the expression of *dnmts* in the hematopoietic compartment at 72h we analyzed the expression of *dnmt4*, *dnmt5*, *dnmt6*, *dnmt7* and *dnmt8* by *in situ* hybridization. The other *dnmts* were not expressed in the CHT region. We also cross sectioned these embryos at 72h to analyze the expression of the transcripts in the dorsal aorta and did not find any significant expression.

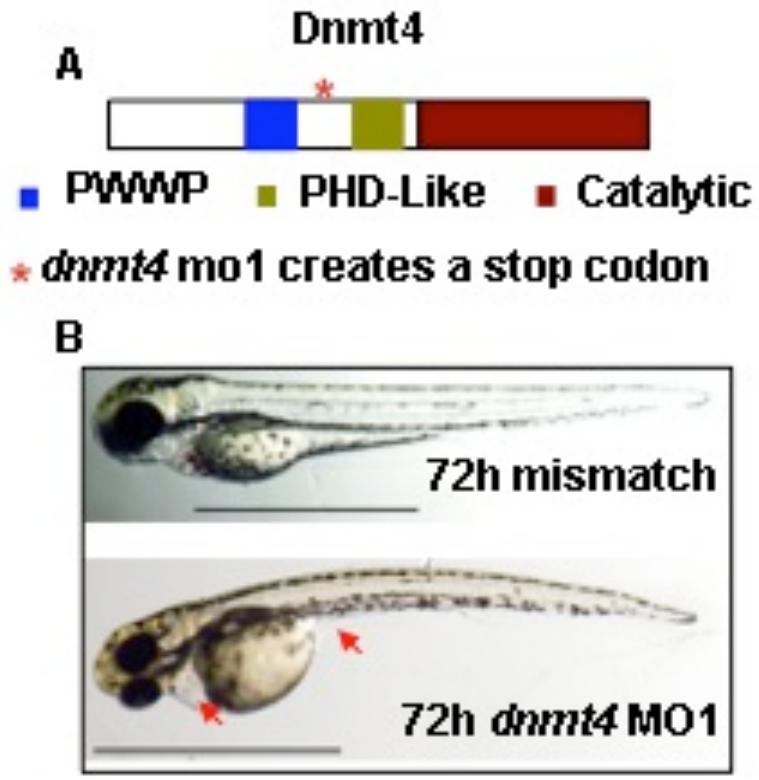
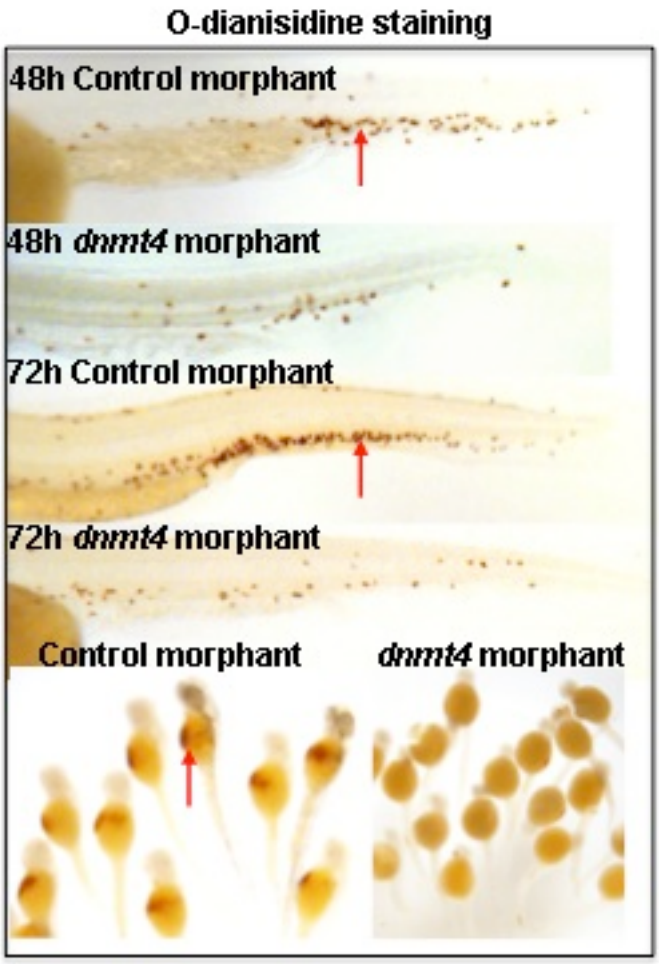
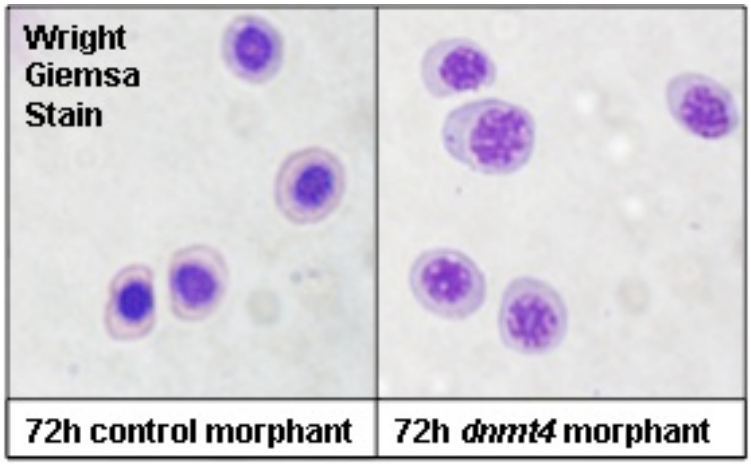


Figure 3.4. Knock down of *dnmt4.* (A) The domain structure of dnmt4. The asterisk denotes the inclusion of a stop codon in the protein on translation of the unspliced product. (B) The gross morphological defects observed in the *dnmt4* morphants at 72h in comparison to the mismatch morpholino.

Figure 3.5. Transient knock down of *dnmt4* affects onset of erythroid differentiation in zebrafish embryos at 48h and 72h.Correct maturation or hemoglobinization of the embryonic erythroid cells does not occur in *dnmt4* morphants: The onset of erythrocyte differentiation is analyzed by o-dianisidine staining. Dnmt4 morphants at 48h and 72h show anemia and decreased somitic iron stores. (Lower panel) Peripheral blood smear of zebrafish embryos at 72 hours shows more immature cells as seen by increased nucleus to cytoplasm ratio in comparison to the control morpholino. Also, the cytoplasm of the *dnmt4* morphants show lesser hemoglobinization of the cytoplasm (bluish color of the cytoplasm) in comparison to the reddish color of the cytoplasm in the control morphants. Note the difference in the decondensed nuclei of the *dnmt4* morphants peripheral blood cells.


O-dianisidine staining
48h Control morphant
48h dnmt4 morphant
72h Control morphant
72h dnmt4 morphant
Control morphant
dnmt4 morphant


Wright
Giemsa
Stain
72h control morphant
72h dnmt4 morphant

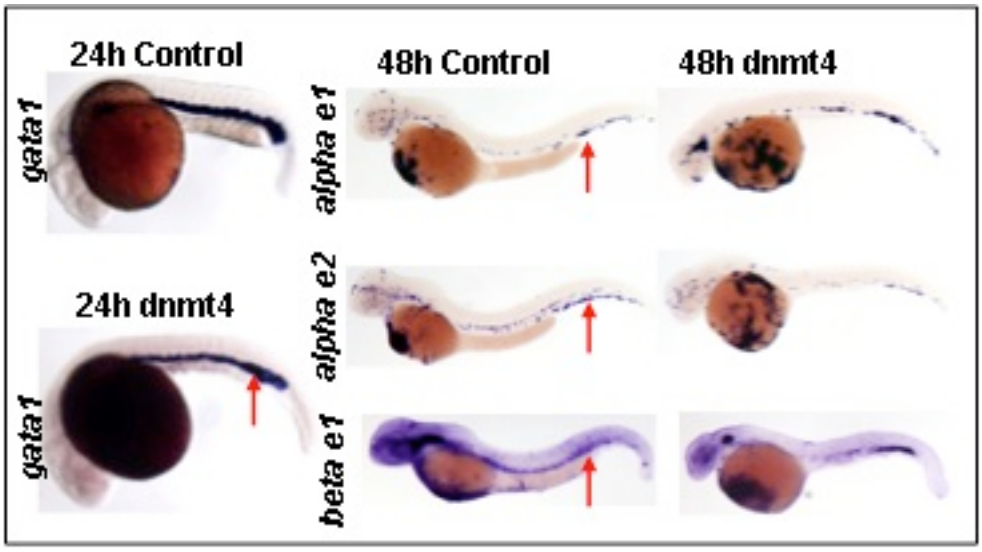
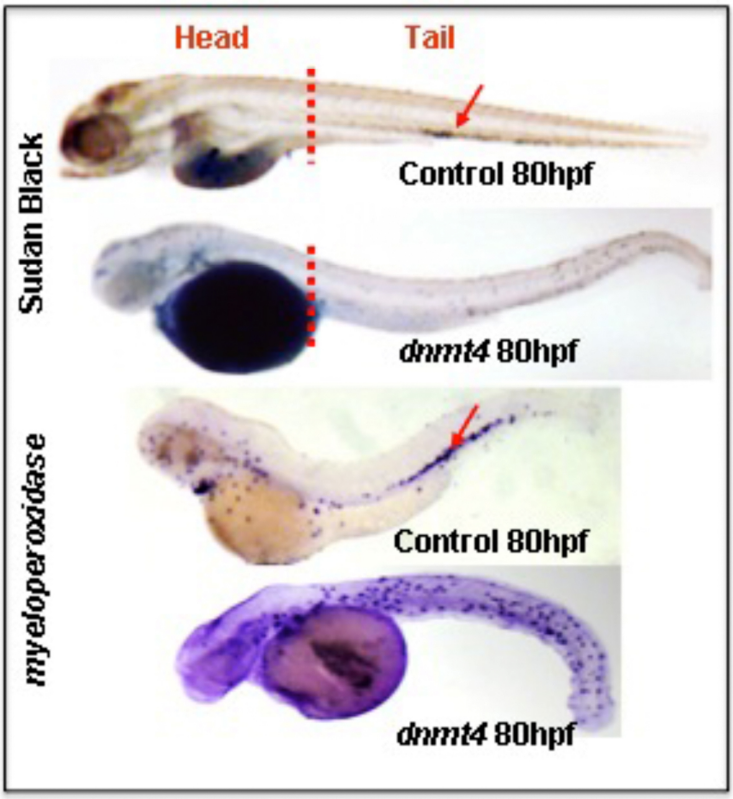


Figure 3.6. Specification of erythroid lineage is unaltered in *dnmt4* morphants. Transient knock down of *dnmt4* affects onset of erythroid differentiation in zebrafish embryos at 48h and 72h but not specification of the erythroid lineage and formation of progenitors. RBC specification (*gata1*) and progenitor formation (*globins*) are unaffected in *dnmt4* morphants

Figure 3.7. Transient knock down of *dnmt4* causes reduction of mature lipophilic neutrophils Sudan Black histochemical staining was used for detecting lipophilic mature neutrophils in *dnmt4* morphants. The sudan black positive cells are down regulated in the *dnmt* morphants embryos in comparison to the 80h control embryos (upper panel and lower graph). W- wild type embryo and D4 –dnmt4 morphant embryo. However, the immature neutrophils were expressed in the *dnmt4* morphants as assessed by the expression of *mpo* positive cells. Notably the *mpo* positive cells were mislocalized from the CHT region.


Head
Tail
Sudan Black
Control 80hpf
dnmt4 80hpf
myeloperoxidase
Control 80hpf
dnmt4 80hpf

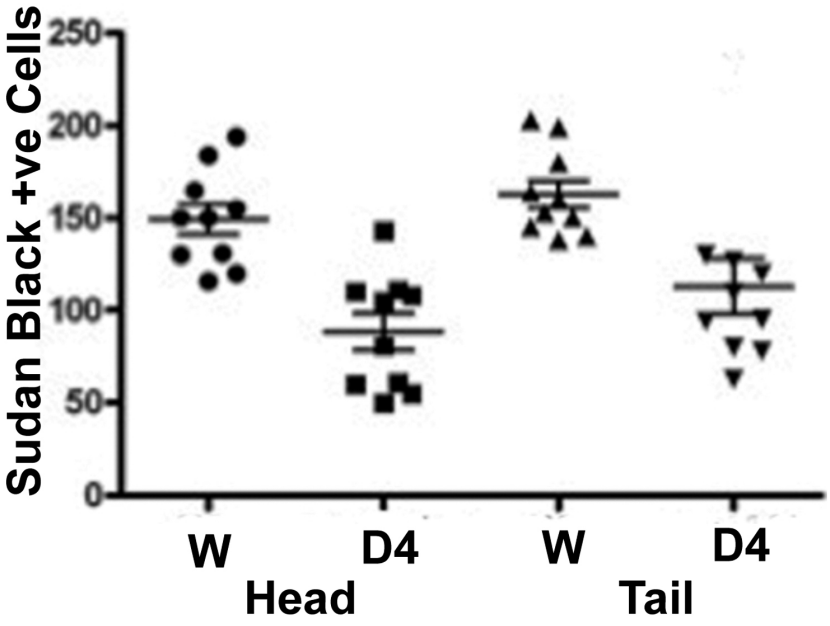
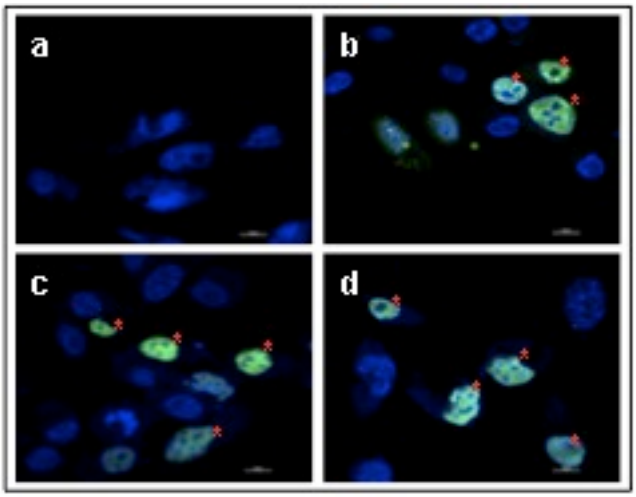

Sudan Black +ve Cells
250
200
150
100
50
0
W
D4
W
D4
Head
Tail

Figure 3.8. Rescue of the gross morphological phenotypes of the *dnmt4* morphants at 72h. The vectors encoding the human proteins used to rescue the *dnmt4* mo1 defects are expressed correctly and localize to the nucleus of the cells in culture (Upper Panel) Overexpression of vectors used for rescue experiment. SW480 cells were (a) untransfected or tranfected with (b) pCMV6-AC-GFP clone containing DNMT3A transcript variant1 (c) pcDNA-DEST53 vector containing DNMT3B and (d) pcDNA-DEST53 vector containing DNMT3B catalytic mutant. The cells were transfected with the respective clones after 24h of plating. After 48h of transfection IHC was performed on the cells and washed and imaged with 60X oil immersion lens using a Nikon A1R/A1 confocal microscope. DAPI – Blue. Over-expressed exogenous DNMT3A, DNMT3B and DNMT3B catalytic mutants in Green. (Lower Panel) *Dnmt4* is a functional homolog of the human *DNMT3B*. *Dnmt4* morphants (2) show smaller heads, mircophthalmic eyes and yolk tube malformations that are rescued by co-injection of catalytically active *dnmt4* (4), human *DNMT3B* (5) and not by catalytically inactive zebrafish *dnmt4* (3) catalytically inactive human *DNMT3B* (6) and human *DNMT3A*.


a
b
c
d


1
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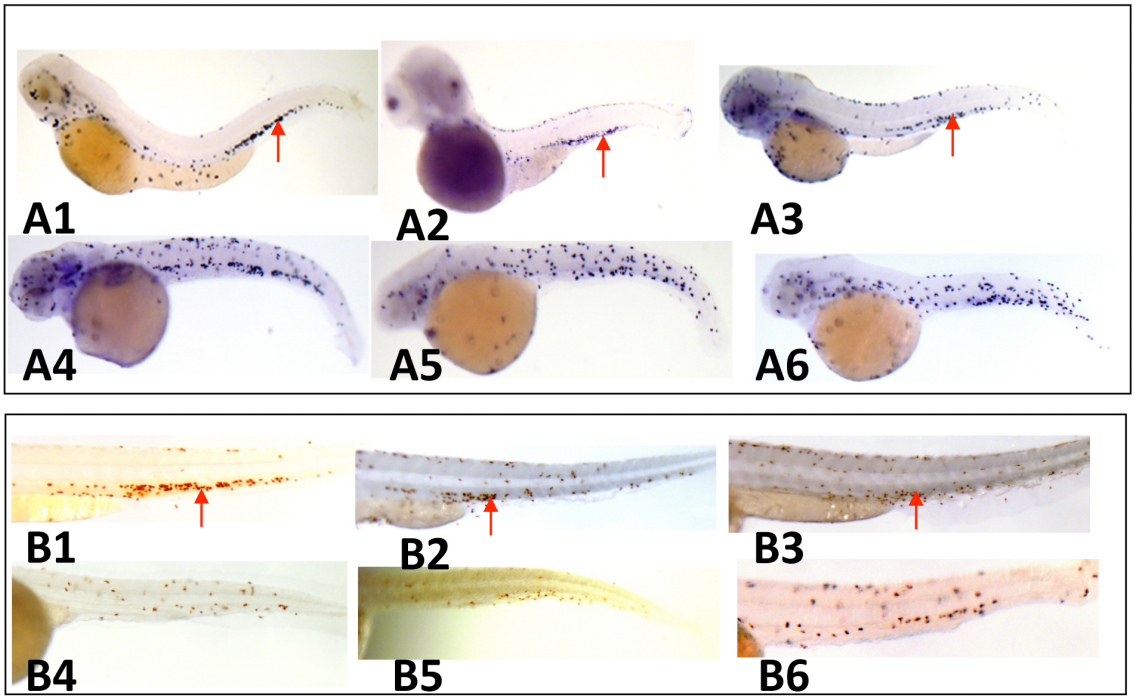

Figure 3.9. Rescue of the *mpo* misexpression and o-dianisidine staining in the *dnmt4* morphants with injection of *dnmt4* RNA and DNMTB. Upper panel shows *mpo* expression and lower panel shows o-dianisidine staining. A1, B1 control morphants, A4, B4- *dnmt4* morphants, A2, B2-*dnmt4* morphants + *dnmt4* RNA, A5, B5- *dnmt4* morphants + mutant *dnmt4*, A3, B3-*dnmt4* morphants + DNMT3B, A6- B6-*dnmt4* morphants + mutant DNMT3B.

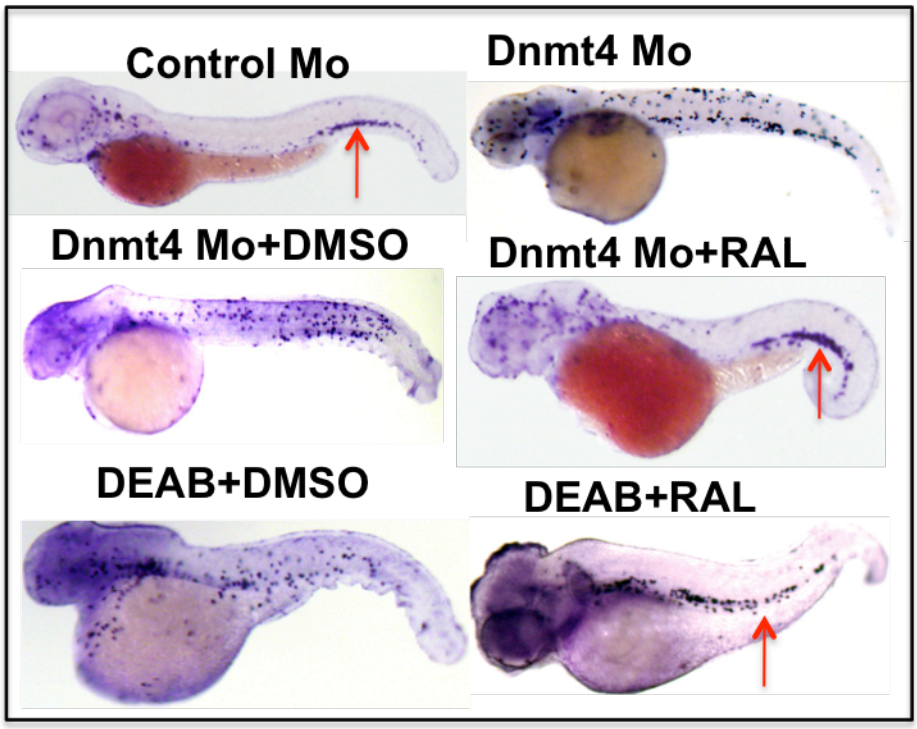

Figure 3.10. Treatment of *dnmt4* morphants with retinaldehyde rescues the misloclization of *mpo* positive cells. Treatment of control morphants with RAL does not alter the distribution of *mpo.* (95%, n=45), data not shown. Treatment of DEAB alters *mpo* distribution like dnmt4 morphants (5%, n=55). Mislocalization of mpo cells in DEAB treated fish is rescued by treatment with RAL (45%, n=120). The *dnmt4* morphants (Dnmt4 mo1) shows altered *mpo* mislocalization (10%, n=50), which is rescued by treatment with RAL shown in middle panel (rescue 50%, n=80). The % represents the embryos showing WT stain. The total number of embryos is n.

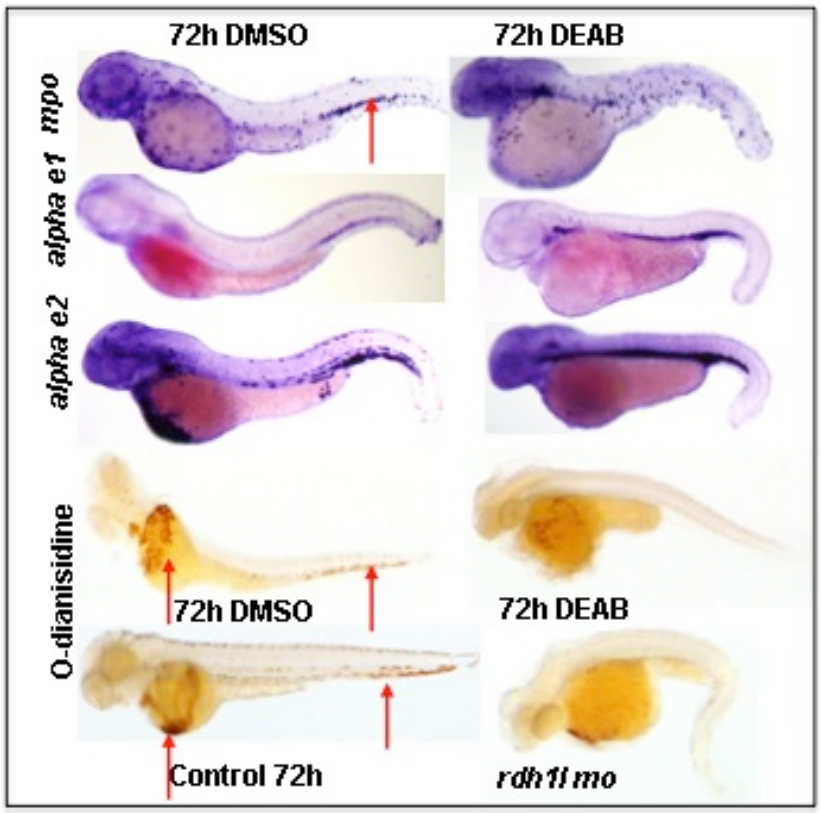

Figure 3.11. Retinoic acid deficiency phenocopies erythroid hemoglobinization and *mpo* defects seen in *dnmt4* morphants. Retinoic acid deficient fish created by pharmacologically blocking retinoic acid synthesis with DEAB an inhibitor of *raldhs* in zebrafish leads to erythroid hemoglobinization defects (95%, n=55). However, the expression of *alphae1*, *alphae2*, are significantly unaltered (100% embryos show WT stain, n=25 each). The DEAB treated embryos display similar mislocalization of the *mpo* positive cells (95%, n=50). Retinoic acid deficiency created by knocking down the levels of *rdh1l* (60%, n=35) causes similar reduction in o-dianisidine positive cells.

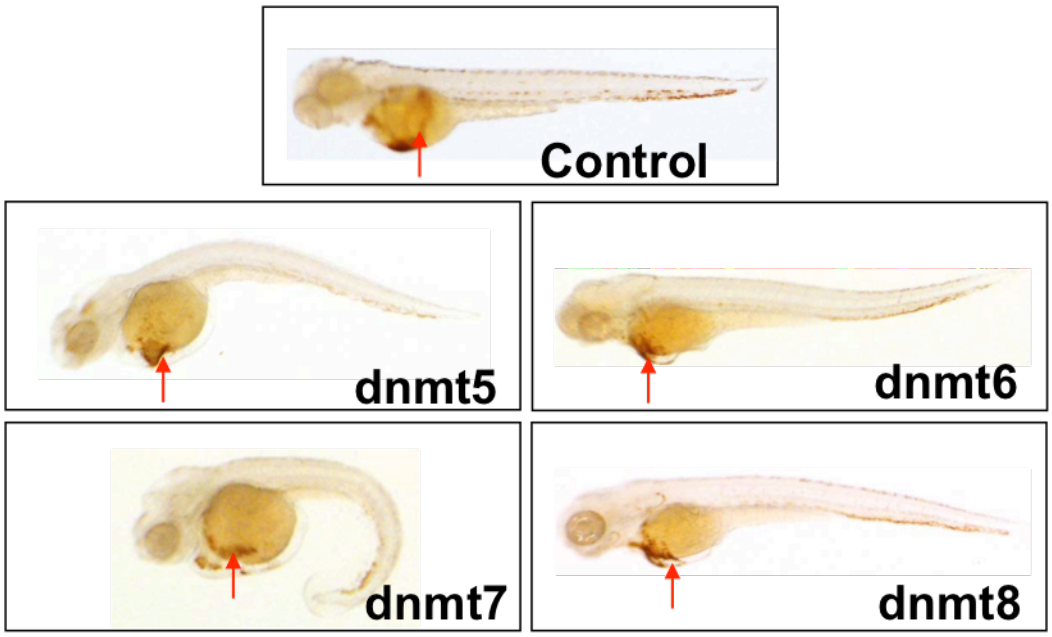

Figure 3.12. The expression of o-dianisidine positive cells in other *dnmt* morphants at 72h. On investigating the expression of o-dianisidine positive cells in *dnmt5* (70%, n=30), *dnmt6* (100%, n=30), *dnmt7* (72%, n=35) and *dnmt8* (100%, n=25) morphants we saw that the expression of o-dianisidine positive cells was significantly unaltered in comparison to the control morphants. The expression of o-dianisidine positive cells in *dnmt4* morphants at this stage was apparently reduced form the CHT region on heart region.

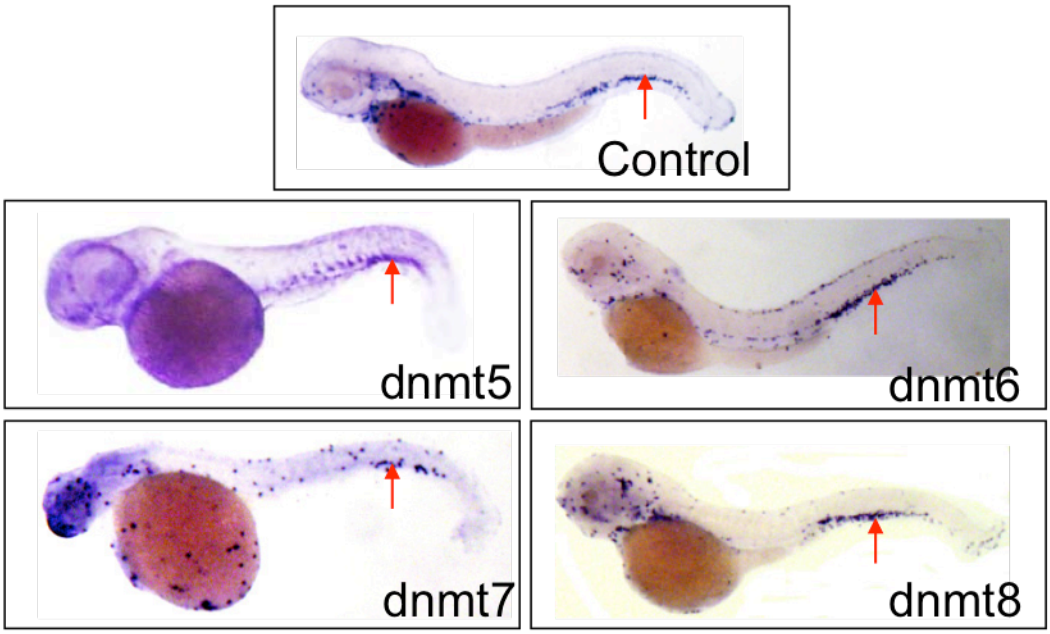

Figure 3.13. The expression of *mpo* in other *dnmt* morphants at 72h. On investigating the expression of myeloperoxidase in *dnmt5* (75%, n=30), *dnmt6* (100%, n=25), *dnmt7* (70%, n=25) and *dnmt8* (100%, n=20) morphants we saw that the expression of *mpo* was significantly unaltered in comparison to the control morphants. The expression of *mpo* in *dnmt4* morphants at this stage was apparently mislocalized form the CHT region. The percentages represent the embryos showing wild type expression. The total number of embryos is n.

Table 3.1. Morpholino and primer sequences.

Gene	Splice Junction	Morpholino Sequence
dnmt4	e9i8	AGCCACCTGAGGACACACAGGAAGC
dnmt4	e7i8	AGGCATTGTATTTTAGTTACCTCTG
dnmt5	e2i2	TGTCTTTTACCCCTTACCTAGCATC
dnmt6	e5i4	TGGCTACCTGTAAGACAAGACAGTC
dnmt7	e3i2	TGCACCTAAAAACAAGACAAAACAG
dnmt8	e12i13	ACATAATCTGAACTTACAAAGTCCT
rdh1l	e2i3	TCTGTCAGTGACTCACCCTTCTGTC
p53	e18i19	TAGCATACTCTACCTGTGCTCTTCG

Primers.

Gene	Forward Primer-1	Reverse Primer-1
dnmt4-1	GATAATAAGGGCTTTGGTA TTGG	CTCAAGGGCCTGGAAGATC
dnmt4-2	ACACACTGACAACATCAAG AG	tcagttcctcaacagcctat
dnmt5	ATTCAGGTGGATCCAGGC	CTGTTGCCTTTGAGTTCCTTC
dnmt6	cattttattctgttgtctacatattgag	CTCTGTCACTTCTAAATCCC
dnmt7	GTGTTACTGTGTGGAGTGT GTGG	CTGTAGCAATGCCATCAAATAAA GACAG
dnmt8	GTCATCGCTGGCTGCTAC	CTCCAAGAGTACTGATCCTGC
rdh1l	CGAAGCCAAACCAACACC	GCGTTGCTCACTATCGTC
apc		

	Reverse Primer-2	Forward Primer-2
dnmt4-1		
dnmt4-2	Cgtgattctgattggctgatg	
dnmt5	TGTCCAAATCCGACACCG	
dnmt 6		ATGGCTACAAATGTTAGTCTGG
dnmt7		ATGGCTACAAATGTTAGTCTGG
dnmt8	Cattcttgctgtgaggcaacagtg	
rdh1l	gtcatagagcctgtactggc	
apc	catcacaccaatcaccacag	

Table 3.2. Statistics of the *dnmt4* morphants.

	Control Mo	***Dnmt4 Mo***	***Dnmt4 Mo + dnmt4^{WT}***	***Dnmt4 Mo + dnmt4^{cm}***	***Dnmt4 Mo + DNMT3B^{WT}***	***Dnmt4 Mo + DNMT3B^{cm}***
gata1	100% n$^{\$}$=30	95% n=40	n/a	n/a	n/a	n/a
alphae1	100% n=45	97% n=60	n/a	n/a	n/a	n/a
alphae2	100% n=50	95% n=45	n/a	n/a	n/a	n/a
betae3	100% n=40	98% n=40	n/a	n/a	n/a	n/a
o-dianisidine	100% n=60	10% n=200	50% n=80	3% n=40	55% n=90	4% n=45
mpo	100% n=65	10% n=150	55% n=70	5% n=30	52% n=65	10% n=40
Sudan Black	100% n=84	3% n=96	n/a	n/a	n/a	n/a
Alcian Blue	100% n=60	20% n=80	57% n=21	10% n=35	60% n=50	13% n=40

Values shown indicate the percentage of n embryos staining positively for the indicated marker. $^{\$}$n is the total number of embryos used in two or three different replicates of an experiment. Zebrafish genes are annotated in lowercase font while human genes are annotated in uppercase font.

3.6 References

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CHAPTER 4

EPISTATIC RELATIONSHIP BETWEEN DNA METHYLTRANSFERASE 4 AND ADENOMATOUS POLYPOSIS COLI

4.1 Introduction

Adenomatous polyposis coli (APC) is a large (310kDa) protein that has multiple functions in maintaining cellular homeostasis. *Apc* functions as a tumor suppressor and is also involved in cell adhesion, microtubule formation, regulating cell cycle and stability of chromosomes (1). Nearly 85% of all sporadic colon cancer cases have mutations in both the alleles of the *APC* gene (2). In the hereditary form of colon cancer, familial adenomatous polyposis (FAP), one mutated *APC* allele is inherited and loss of heterozygosity leads to tumorigenesis.

In addition to the molecular functions of *Apc* in maintaining intestinal homeostasis it is also expressed in the thymus, lymphocytes, and lymphobastic cell lines (3).The most well characterized and extensively used animal model for colon cancer is the $Apc^{Min/+}$ mouse. Importantly, $Apc^{Min/+}$ mice exhibit atrophy of the lymph and thymic nodes at 80 days (4). Around 120 days these mice exhibit a total regression of the thymus. These mice also exhibit depletion of splenic natural killer cells, immature and progenitor B cells suggesting that *Apc* may play

a role in hematopoiesis. However, why these mice start showing thymic atrophy at 80 days is not known. In a separate study, enhanced splenic hematopoiesis was observed at 12 and 15 weeks in *Apc*$^{Min/+}$ mice (5).

Recent studies have also indicated the requirement of *Apc* in proper erythropoiesis (6). Mice with conditional removal of one allele of *Apc* exhibit severe anemia. Erythropoiesis is blocked in these mice at early stages of differentiation. These heterozygous mice have an expansion of long and short-term hematopoietic stem cell populations. Myeloid progenitor cells are apoptotic suggesting that haploinsufficiency of *Apc* leads to impaired hematopoiesis. Also, this data hints at the possibility that loss of function of *Apc* may lead to myelodysplastic syndrome (6).

In a separate study *Apc*$^{Min/+}$ mice were also shown to develop myeloproliferative phenotypes (7). The *APC* gene is located on chromosome 5q, and this region is commonly deleted in myelodysplastic syndromes. Based on these data it is hypothesized that loss of function of *APC* may cause the phenotypes observed in myelodysplastic syndromes.

On deleting both the alleles of *Apc* in the hematopoietic compartment mice displayed severe bone marrow necrosis. The hematopoietic stem and progenitor cells in these mice showed rapid apoptosis, suggesting the requirement of *Apc* in hematopoietic stem and progenitor cell maintenance and survival. Also, these mice displayed ineffective differentiation of the myeloid and the lymphoid populations (8).

The canonical function of APC includes regulating WNT signaling *via* proteasomal degradation of β-catenin (9). In addition to this function, loss of *apc* correlates with up-regulation of a DNA demethylase system and down-regulation of *dnmt1* in the intestine of *apc* mutant zebrafish at 72h (10). Moreover, APC regulates retinoic acid biosynthesis through regulation of CtBP1 (11). Interestingly, up-regulation of the demethylase components in *apc* mutant zebrafish embryos results from the absence of retinoic acid signaling (10). Cumulatively, these studies underscore the genetic interactions between DNA methylation, DNA demethylation, retinoic acid biosynthesis and APC in regulating intestinal cell fating. However, the involvement of these key players in hematopoietic development has not been fully elucidated.

In Chapter 3, I show that *dnmt4* is required for terminal differentiation of the erythroid and the myeloid lineage cells. Briefly, *dnmt4* transcripts are robustly expressed in the hematopoietic compartment of zebrafish embryos. Knockdown of the transcript levels of *dnmt4* using an antisense morpholino causes anemia and neutropenia in the morphant embryos at 72h. Interestingly, recent data suggests that not only are there distinct DNA methylation profiles in the developing human hematopoietic system, but also these epigenetic changes are affected by age (12).

Notably, the loss of *Apc* in murine hematopoietic cells leads to ineffective hematopoiesis affecting both the erythroid and the myeloid populations. However, the exact mechanisms that regulate this phenotype are not known.

Based on the findings in Chapter 3 and the data discussed above I hypothesize that loss of *apc* leads to ineffective hematopoiesis by down-regulation of *dnmt4.*

To test this hypothesis we have used *apc* mutant zebrafish line to show that *apc* mutant zebrafish embryos show a down-regulation of terminally differentiated erythroid and myeloid cells. Notably the *apc* mutant zebrafish embryos show a down regulation of *dnmt4* transcripts in the hematopoietic compartment. The *apc* mutant embryos show mislocalization of *mpo* transcripts, which is rescued by the treatment of modulators of retinoic acid biosynthesis. However, treatment with WNT pathway inhibitor does not rescue this mislocalization, suggesting that the altered distribution of the *mpo* cells was through lack of retinoic acid biosynthesis.

4.2 Materials and methods

4.2.1. Zebrafish maintenance

Zebrafish stocks were maintained in z-mods on a 14:10 h light:dark cycle at 28°C in the Huntsman Cancer Institute zebrafish fish facility using standard fish husbandry IACUC protocols. Fertilized embryos were grown at 28.5°C. For whole mount *in situ* hybridization embryos were raised in 0.003% phenylthiourea to inhibit pigment formation. *apc* mutant zebrafish line was generated in the Clevers lab.

4.2.2. Oligonucleotides and constructs

The GenBank accession number for *dnmt4* is NM_001025450. Zebrafish *dnmt4* was cloned in pCRTOPOII vector. Michael Redd provided clones for *mpx*, *cmyb* and *gata1*, and Sarah Hutchinson provided clones for *alphae1*, *alphae2*, *betae1*, *betae3*. The sequence of the *dnmt4* morpholino has been shown in Chapter 3.

4.2.3. Whole mount *in situ* hybridizations

Zebrafish embryos at mentioned time points were fixed in sucrose buffered 4% para-formaldehyde. The embryos were washed in PBS, dehydrated and stored in methanol at -20°C. Whole mount *in situ* hybridizations were carried out as described previously (13) using digoxigenin-labeled riboprobes for *dnmt4*, *mpx*, *spi1*, cmyb, *gata1*, *alphae1*, *alphae2*, *betae1*, and *betae3.*

4.2.4. O-dianisidine staining

Hb activity was detected in whole embryos by histochemical staining for Hb using o-dianisidine as described previously (14). Briefly, embryos were placed in freshly prepared o-dianisidine stain solution (40% ethanol with 0.65% H2O2, 0.01 M sodium acetate, and 0.6 mg/mL o-dianisidine (D-9143; Sigma) for 20 min and then were washed in water. Stained embryos were fixed in 80% glycerol and imaged.

4.2.5. Sudan Black staining

Sudan Black histochemical staining for detecting lipophilic mature neutrophils was performed as described previously (15). Briefly, embryos were fixed in 4% PBS for 2h at room temperature. Rinsed three times with PBS, incubated in Sudan Black (Sigma-Aldrich) for 20 min. The embryos were washed with extensively with 70% ethanol in water over 2h, then serially rehydrated to PBS and 0.1% Tween 20.

4.2.6. Statistical analyses

Prism 4 (GraphPad Software, San Diego, CA) was used for statistical analyses. Results of statistical analyses are provided in supplementary Table S1. The quantitative data is representative of at least three independent experiments.

4.2.7. Drug treatments

To rescue the retinoic acid deficiency *apc* mutants and control siblings were treated with 0.3μM all-*trans*-retinaldehyde (ATRAL; Sigma-Aldrich) at 10h, 24h, 48h and 72h for 30 min. After each treatment the embryos were washed thrice with embryo water. To preclude the involvement of WNT signaling in the observed phenotypes, embryos were treated with an inhibitor of *cox2* (NS-398) at 10μM concentration 10h. The drug was allowed to remain in the media through 72h. The embryos were allowed to grow until 72h and later fixed for *in situ* hybridization analyses. The *apc* mutant embryos harbor elevated levels of *cyp26a1* a catabolic regulator of retinoic acid. To restore retinoic acid

biosynthesis in the *apc* mutant embryos they were treated with 5μM RAMBA, a pharmacologic inhibitor of *cyp26a1*, the levels of which are upregulated in *apc* mutants (16).

4.2.8. PCR and expression analysis

Total RNA was harvested using Trizol reagent (Invitrogen) according to the manufacturer's protocol. The RNA was treated with DNAse1 to remove gDNA contamination. cDNA for PCR reactions was synthesized from 2 μg of total RNA using Superscript III (Invitrogen). PCR was performed using the Roche Lightcycler instrument and software, version 3.5 (Roche Diagnostics). A template-free negative control was included in each experiment. The sequences of PCR primers used: *aid*, *mbd4*, *gadd45a*, and *28s* have been described elsewhere (10).

4.3 Results

To determine if the *apc* mutant zebrafish embryos exhibit hematopoietic defects we investigated if they harbored terminally differentiated erythrocytes and neutrophil cells. The *apc* mutant zebrafish embryos exhibited sharp reduction in the o-dianisidine positive cells (Figure 4.1. upper panel), suggesting that they are anemic. However, the mRNA for progenitor blood cell populations like *cmyb*, *gata1*, *alphae1*, *alphae2*, *betae1*, and *betae3* were expressed (data not shown).

Notably the *apc* mutants harbored fewer Sudan Black positive cells suggesting that lipophilic mature neutrophils were drastically reduced (Figure 4.1

lower panel); however, the mRNA transcripts for *mpo* were expressed robustly but mislocalized in the *apc* mutant embryos (Figure 4.2.). This suggested that although the progenitors were specified the terminally differentiated cell populations were drastically reduced.

To determine if the hematopoietic phenotypes that were observed in *apc* mutants was due to loss of *dnmt4*, we investigated the expression of *dnmt4* in *apc* mutant embryos. We found that levels of *dnmt4* were down-regulated in the CHT region of the *apc* mutant embryos suggesting that *apc* may regulate the expression of *dnmt4* (Figure 4.3). However, the levels of *apc* were unaltered in *dnmt4* morphants suggesting that *dnmt4* does not regulate the expression of *apc* (Figure 4.4).

To determine if the altered distribution of the *mpo* cells was due to aberrant retinoic acid signaling we treated the embryos with retinaldehyde, RAMBA or the vehicle control DMSO (Figure 4.5). Interestingly the mislocalization of the *mpo* cells was rescued in the *apc* mutant embryos supplemented with RAL and RAMBA but not with NS-398 an inhibitor of *cox2*, suggesting that aberrant retinoic acid signaling and not overactive *wnt* is responsible for the this phenotype.

The rescue of mislocalization of the *mpo* positive cells in *apc* mutants with RAL and RAMBA suggests that retinoic acid is involved in terminal differentiation of erythroid and myeloid cells. To address if *dnmt4* loss causes retinoic acid deficiency we performed quantitative RT-PCR to analyze the transcript levels of *aid*, *mbd4* and *gadd45a* in the *dnmt4* morphants. The *dnmt4* morphants harbored

elevated level of *aid*, *mbd4* and *gadd45a* suggesting that *dnmt4* deficiency leads to retinoic acid deficiency (4.6). However, the mechanisms that cause this retinoic acid deficiency are not known.

4.4 Discussion

The loss of function of *Apc* can lead to impaired functions of HSCs and HSPCs (8). Moreover, mice with heterozygous *Apc* show lethal anemia, monocytosis and macrocytosis between 3 to 8 months (6). Also, these mice exhibit a block in differentiation of erythroid development suggesting that *Apc* function may regulate hematopoiesis. Importantly, this anemia was not due to loss of blood, or vitamin deficiency suggesting that these cells failed to terminally differentiate into mature erythroid cells.

Moreover, it is not understood how these erythropoietic defects arise in these mice between particular ages that were reported. The DNA methylation profiles of cells that are arrested in differentiation are not known. Our data supports the notion that *apc* levels regulate *dnmt4* expression in the hematopoietic compartment. However, if *apc* affects the transcription, translation or stability of *dnmt4* is not known. Also, *dnmt4* loss leads to retinoic acid deficient phenotypes, which can be rescued by retinoic acid supplementation. This is also corroborated by the rescue of *mpo* mislocalization in *apc* mutant zebrafish embryos. *apc* mutant zebrafish embryos are retinoic acid deficient (10,11,17), suggesting that retinoic acid may indeed play a role in regulating hematopoietic differentiation. However, it has still not been investigated if retinoic acid pathway

is directly regulated by *dnmt4*. Also it is not known if, retinoic acid regulates the expression *of dnmt4*. Figure 4.7 shows models that may explain the epistatic relationships between *apc*, *dnmt4* and retinoic acid.

4.5 Acknowledgements

I would like to thank Michael Redd and Sarah Hutchinson for sharing probes and protocols for this study.

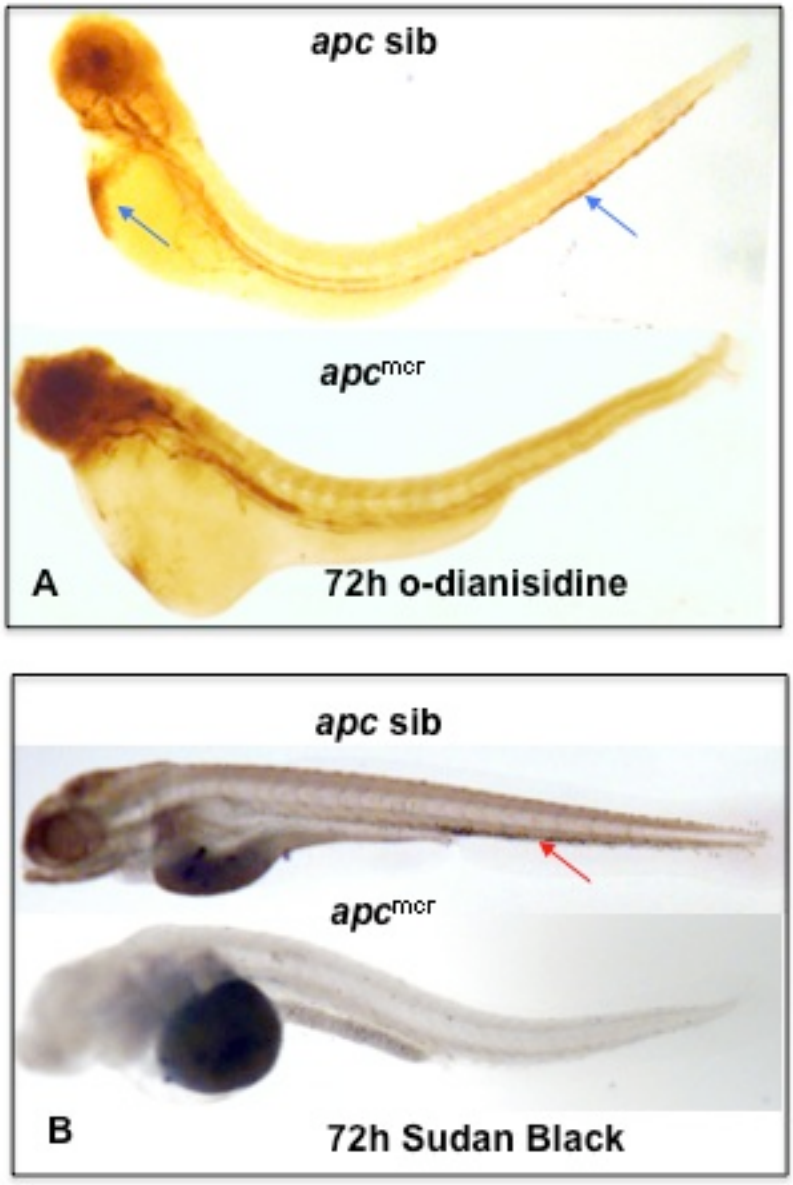


Figure 4.1. *apc* mutant zebrafish harbor anemia and neutropenia at 72h. (A) *apc* mutant zebrafish show reduced staining for o-dianisidine suggesting that they suffer from anemia. (B) The *apc* mutant embryos exhibit a drastic reduction of lipophilic mature neutrophils as seen by Sudan Black staining.

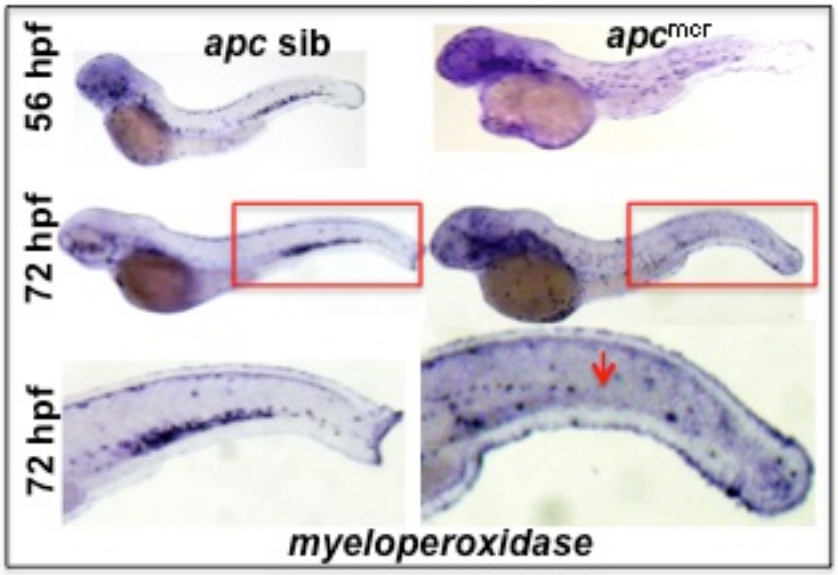


Figure 4.2. Transcripts for immature neutrophils (*mpo* positive cells) are expressed but their distribution is altered in the *apc* mutant embryos. At 56h and 72h the apc mutant embryos exhibit an altered distribution of *mpo*. The transcripts are mislocalized to regions other than the CHT (as shown in the inset of the CHT region. However, it is not known what causes the mislocalization of these cells from the CHT and why there are reduced numbers of lipophilic mature neutrophils in the mutants.

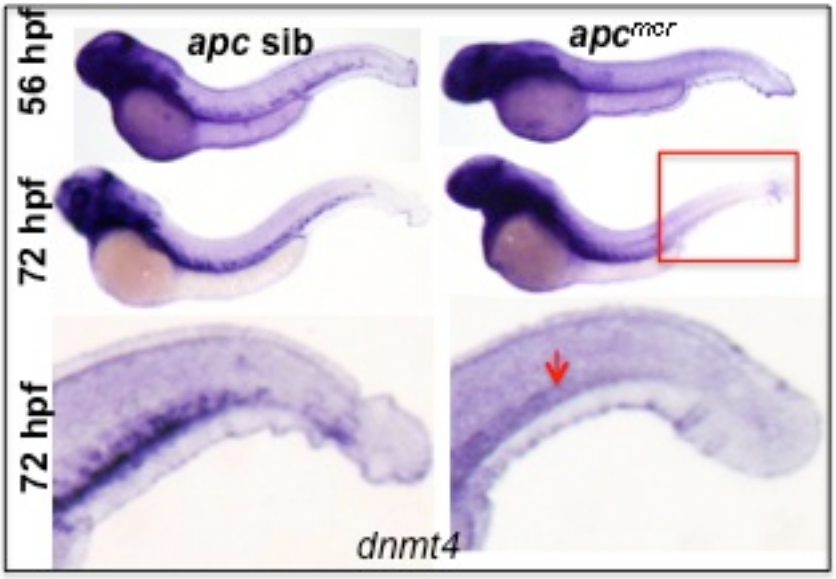


Figure 4.3. The transcripts of *dnmt* are drastically reduced in the CHT region of the *apc* mutant embryos. Zebrafish *dnmt4* transcripts are drastically down regulated in the CHT region of *apc* mutant embryos both at 56h and 72h. The inset shows the expression of *dnmt4* in the CHT region at 72h in the mutant. It is not known what causes this downregulation of the *dnmt4* transcripts.

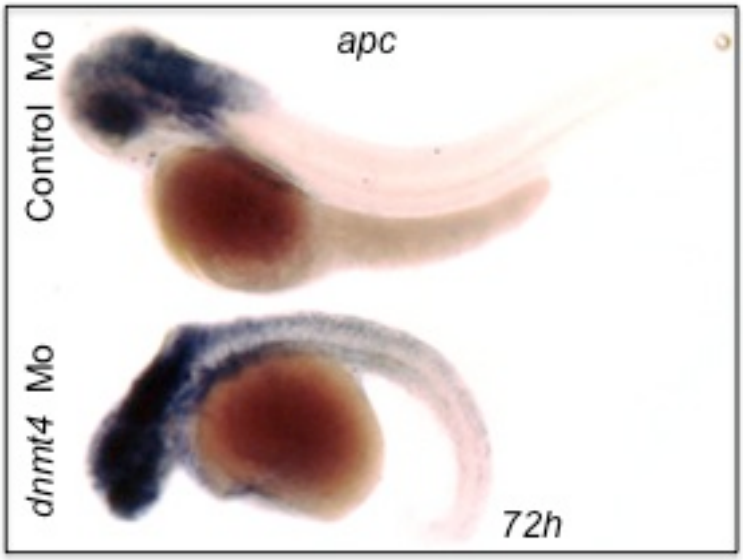


Figure 4.4. The transcripts of *apc* are unaffected in the *dnmt4* morphants. In the zebrafish *dnmt4* morphants the transcripts of *apc* are unaffected suggesting that the *dnmt4* does not control the expression of *apc* during embryonic development.

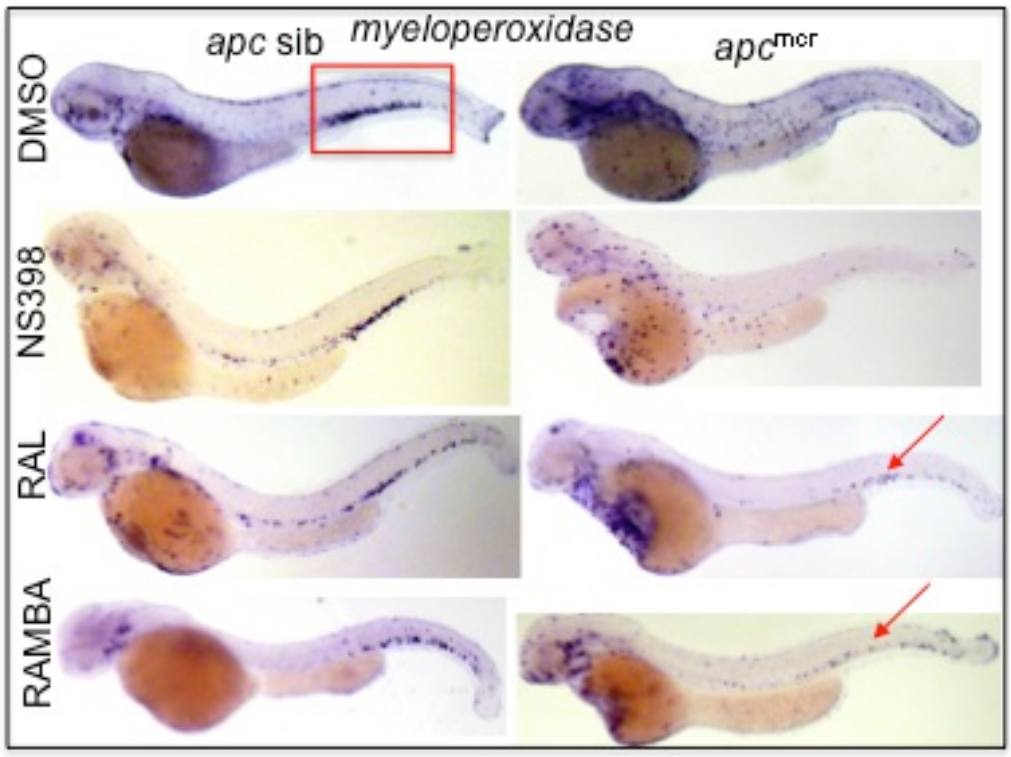
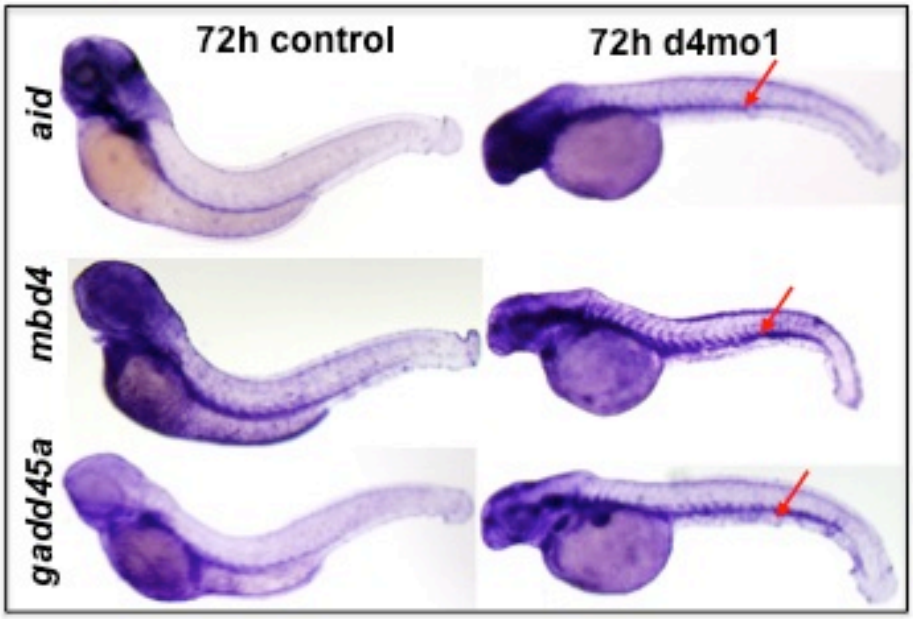
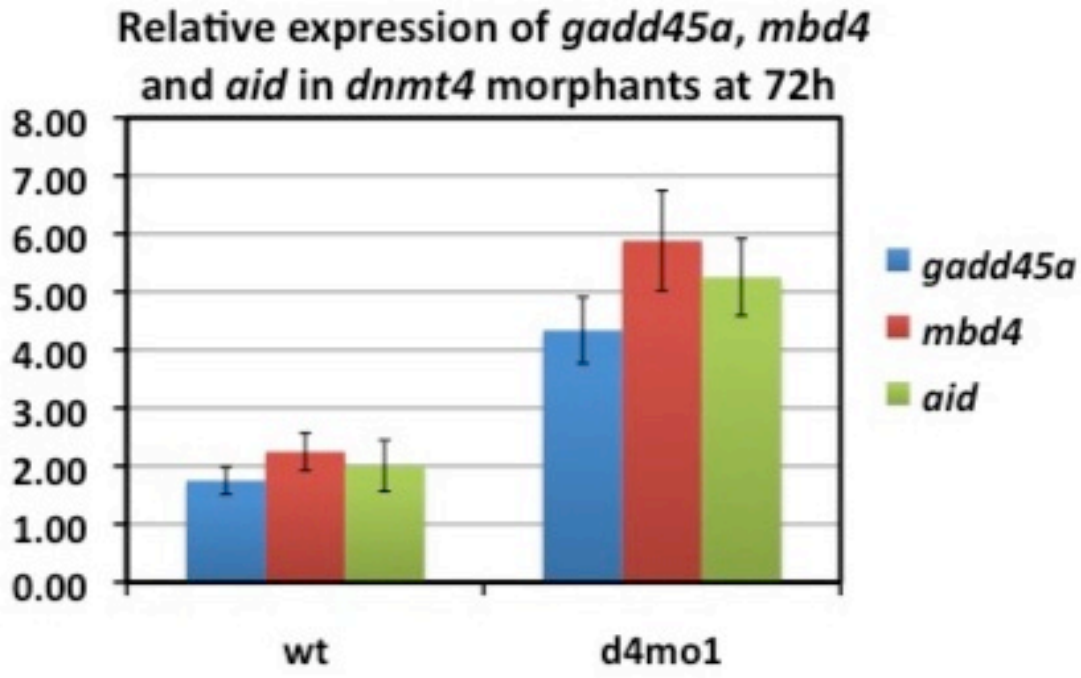


Figure 4.5. The mislocalization of *mpo* positive cells in *apc* mutant embryos is rescued by pharmacological rescue of the retinoic acid pathway. At 72h the *apc* mutant embryos exhibit an altered distribution of *mpo*. The transcripts are mislocalized to regions other than the CHT. The exogenous supplementation of retinaldehyde and RAMBA (inhibitor of RA catabolic enzyme *cyp26a1*) rescues the mislocalization of *mpo* positive cells, suggesting a role of retinoic acid signaling in this process. However, inhibiting the over-active *wnt* signaling with supplementation of *cox2* inhibitor NS-398 does not rescue this mislocalization suggesting this phenomenon is not regulated by *wnt* signaling.

Figure 4.6. Loss of *dnmt4* in wild embryos with an antisense morpholino causes up-regulation of demethylase complex. Zebrafish *dnmt4* downregulation using an antisense morpholino causes upregulation of members of the demethylase complex in zebrafish *aid*, *mbd4* and *gadd45a* as seen by *in situ* analyses of 72h *dnmt4* morphants in the *upper* panel and by quantitative RT-PCR analyses of the *aid*, *mbd4* and *gadd45a* transcripts relative to the expression of 28S transcripts.


72h control
72h d4mo1
aid
mbd4
gadd45a


Relative expression of gadd45a, mbd4 and aid in dnmt4 morphants at 72h
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
wt
d4mo1
gadd45a
mbd4
aid

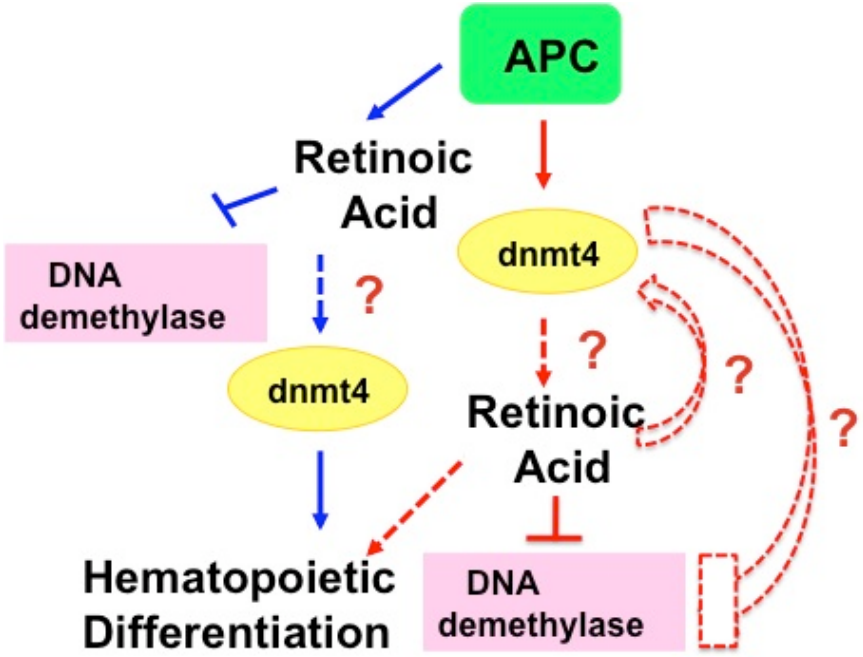


Figure 4.7. Putative models that represent the epistatic relationships between *dnmt4*, *apc*, and the retinoic acid pathway. The *dnmt4* morphants harbor hematopoietic defects that are rescued by supplementation of retinoic acid. However, if retinoic acid affects the transcripts of *dnmt4* is not known. *apc* mutants harbor similar hematopoietic defects to *dnmt4* morphants. The transcripts of *dnmt4* are down-regulated in the *apc* mutants. How *apc* affects the transcripts of *dnmt4* is not known. The *apc* mutants and *dnmt4* are both retinoic acid deficient. However the exact mechanisms that lead to retinoic acid deficiency in *dnmt4* morphants are not known. The model suggested here has incorporated data shown in this chapter and general themes proposed elsewhere (10,11,13,17,18).

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Table 4.1: Statistics of *apc* mutant phenotypes

Marker	Control	*apc* mutant
dnmt4	100% n=50	20% n=60
mpo	100% n=40	5% n=60
Sudan Black	100% n=50	4% n=55
o-dianisidine	100% n=30	5% n=50

Values shown indicate the percentage of n embryos staining positively for the indicated marker. n is the total number of embryos used in two or three different replicates of an experiment.

Table 4.2. Statistics of *apc* mutant *mpo* rescue.

Treatment	*Control Mo*	*apc* muntant
DMSO	100%	5%
	n=40	n=55
RAMBA	100%	55%
	n=35	n=60
RAL	100%	58%
	n=32	n=60
NS-398	100%	7%
	n=45	n=40

Values shown indicate the percentage of n embryos staining positively for the indicated marker. n is the total number of embryos used in two or three different replicates of an experiment.

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CHAPTER 5

SUMMARY AND PERSPECTIVE

5.1 Tissue-specific functions of *dnmt1*, *dnmt3* and *dnmt4*

DNA methylation is a reversible and heritable mechanism to silence genes. However, the examples of differentially methylated gene promoters during embryonic development are few (1, 2). It is hypothesized that DNA methylation occurs in a tissue-specific manner during embryonic development to confer cell-specificity and cell-identity. However, examples of tissue-specific functions of DNA methyltransferases are largely unexplained. This specificity may perhaps arise in part because of the interacting partners of DNA methyltransferases that recruit them to different promoters to silence genes in cell- and tissue-specific manner.

Identifying the target tissues of the different DNA methyltransferases, their interacting partners and their genetic targets will be important in discovering specific inhibitors of these enzymes. DNA methyltransferases may regulate common as well as discrete genes. Thus, to develop specific drugs it is important to identify both common and discrete molecular targets of these enzymes.

The data presented in this thesis supports the idea that there are both discrete and common targets of the DNA methyltransferases (Figure 5.1). Using

an antisense morpholino approach we show that knock-down of *dnmt1* (3), *dnmt3* (4), and *dnmt4* affects terminal differentiation of the exocrine pancreas and the retinal pigmented epithelium. However, terminally differentiation of the intestine (3), brain (4) and blood development are only affected in *dnmt1*, *dnmt3*, and *dnmt4* morphants respectively. This suggests that these three enzymes affect the development of both similar and discrete organs. However, the factors that govern this tissue-specificity are not known. Importantly the genetic targets of each of these enzymes in the affected organs are still unknown. Also, the other DNA methyltransferases - *dnmt5*, *dnmt6*, *dnmt7* and *dnmt8* do not harbor similar hematopoietic defects like *dnmt4* morphants suggesting a role of tissue-specific development.

5.2 Dnmt4 regulates zebrafish hematopoiesis

Stage specific methylation patterns have been shown to regulate gene expression in hematopoietic development (1). However, there is inconclusive data regarding the contributions of different DNA methyltransferases that may regulate hematopoietic lineages (5-7). In the present study we have identified that *dnmt4* is a functional homolog of the mammalian DNA methyltransferase 3B. *dnmt4* morphants partly phenocopy defects seen in the ICF syndrome occurring due to missense mutations in DNMT3B. *dnmt4* regulates terminal differentiation of the erythroid and the myeloid blood cells. However, the molecular targets of *dnmt4* in these cells are still not known.

5.3 Epistatic relationship between *dnmt4* and retinoic acid pathway

The findings reported in this study indicate that there is an epistatic relationship between *dnmt4* and the retinoic acid pathway. The hematopoietic defects observed in *dnmt4* morphant embryos are rescued by the exogenous supplementation of retinoic acid. Moreover, both genetic and pharmacologic knock-down of retinoic acid biosynthesis phenocopy hematopoietic defects seen in *dnmt4* morphants. Interestingly, all-trans retinoic acid is administered as therapy for overcoming the differentiation block in acute promyelocytic leukemia cells (8).

However, the molecular events that regulate this rescue of differentiation block are not known. Important unanswered questions include- how does DNA methylation by *dnmt4* regulate retinoic acid pathway? How does retinoic acid treatment recue defects observed in *dnmt4* morphants? Does retinoic acid treatment induce the expression of *dnmt4*? Is there a feed-back loop between *dnmt4* and the retinoic acid pathway? Answers to these questions are important with respect to the clinical use of DNA hypomethylating drugs (9) in myelodysplastic syndrome and retinoic acid-based therapy in hematological malignancies.

5.4 Epistatic relationship between *dnmt4* and *apc*

APC is a large protein with multiple functions that regulate cell fate commitment. Our data suggests that *apc* positively regulates *dnmt4* expression

in the hematopoietic tissues of the zebrafish embryos. *apc* mutant zebrafish embryos harbor similar hematopoietic defects as the *dnmt4* morphants. Interestingly, the levels of *apc* are unaltered in the *dnmt4* morphants. In our future experiments we will determine how *apc* regulates the expression of *dnmt4.* Also, investigations will be focused at determining how *apc* regulates the chromatin architecture *via dnmt4* in the hematopoietic cells.

5.5 Future directions

In summary, the important findings that have emerged from this thesis work are: 1) Zebrafish DNA methyltransferases 4, 5, 6, 7 and 8 are *de novo* DNA methyltransferases; 2) they have significant over-lapping yet discrete expression patterns in embryonic stages; 3) knockdown of the *dnmt4*, *dnmt5*, *dnmt6*, *dnmt7* and *dnmt8* confers different gross phenotypic defects in these morphants that are distinct from each other; 4) *dnmt4* morphants display cranio-facial and hematopoietic defects, some of which are also seen in ICF patients, 5) the defects seen in *dnmt4* morphants can be rescued with catalytically active version of *DNMT3B* but not the inactive derivative; 6) *dnmt4* morphants do not harbor gut and brain defects like *dnmt1* and *dnmt3* morphants. *dnmt1* and *dnmt3* morphants phenocopy *dnmt4* morphants in harboring hematopoietic defects; 7) *dnmt5*, *dnmt6*, *dnmt7*, and *dnmt8* do not harbor hematopoietic defects like *dnmt4* morphants; 8) retinoic acid deficiency in zebrafish phenocopies hematopoietic defects seen in *dnmt4* morphants; 9) exogenous supplementation of retinoic acid rescues hematopoietic defects harbored in *dnmt4* morphants; 10) *dnmt4* levels

are down-regulated in the CHT region of *apc* mutant zebrafish embryos, and *apc* mutants display similar hematopoietic defects seen in *dnmt4* morphants.

The three main themes that have emerged from the current dissertation work are: first, there is a requirement of DNA methyltransferases both in a distinct and overlapping tissue-specific manner to regulate differentiation, second, there is an epistatic relationship between DNA methylation and retinoic acid signaling; third, *apc* positively regulates DNA methylation.

The future plans for this study include parsing the above observations and themes. We plan to investigate the molecular functions of the different DNA methyltransferases in detail using loss-of-function approaches. We will identify the tissue-specific functions of *dnmt5*, *dnmt6*, *dnmt7* and *dnmt8*. Complementation studies in conjunction with loss-of -function will help us to identify the homology between the zebrafish and human DNA methyltransferases. Also, we will investigate the homology between zebrafish genes and human splice variants. This will help us in deciphering the functions of the multiple isoforms of human DNMT3A and DNMT3B.

We will further identify the genetic targets of the different DNA methyltransferase in the specific organs by utilizing transgenic zebrafish lines to sort specific cell populations for mapping the methylome and transcriptome of the affected tissues in morphants. However, we still do not know the functions of the different DNA methyltransferases in the adult zebrafish. We also plan to investigate these questions. A detailed understanding of the tissue-specific

requirements of the different DNA methyltransferases will help us in designing better therapeutics against DNA methyltransferases.

To determine the molecular targets of *dnmt4* in hematopoietic cells we will determine the transcriptome and methylome of myeloid lineage cells isolated from *dnmt4* injected Tg:*pu.1*:GFP embryos. The other affected lineage in *dnmt4* morphants is the erythroid lineage. We will isolate the erythroid precursor cells from Tg:globin LCR:GFP embryos to determine the molecular targets of *dnmt4* in the red blood cell lineage cells.

Also, as proof of principle, to determine if DNA methylation conferred by DNMT3B is necessary for the terminal differentiation of the erythroid lineage cells, we will knock-down the levels of *Dnmt3b* in MEL (mouse erythro leukemia) cells. The *Dnmt3b* deficient MEL cells should not terminally differentiate into red blood cells in comparison to the control cells. Also, if retinoic acid signaling is deficient in the *Dnmt3b* deficient MEL cells then supplementation of exogenous retinoic acid will rescue the differentiation defects of these MEL cells. If DNA methylation conferred by *DNMT3B* in the erythroid cells is a conserved mechanism then as a proof of principle *Dnmt3b* deficient MEL cells will recapitulate the erythropoietic defects observed in zebrafish *dnmt4* morphants.

We plan to pursue rigorous characterization of the *dnmt4* morphants as a vertebrate model for ICF syndrome. Important future directions include characterizing the cytogenetic abnormalities and other molecular defects in detail in the *dnmt4* morphants.

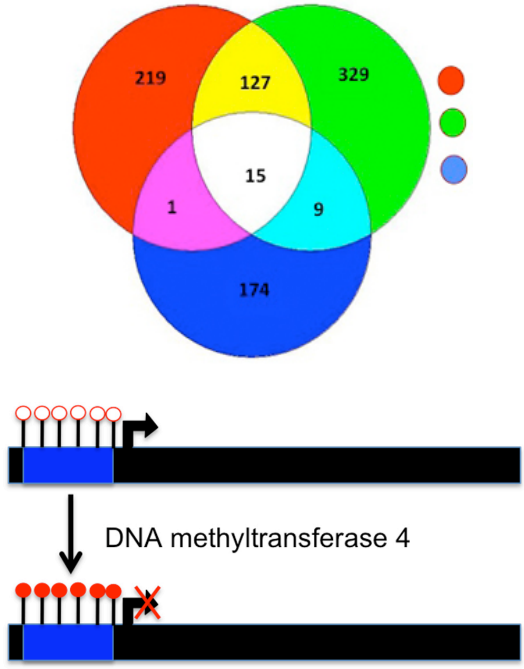

Figure 5.1. Model showing *dnmt4* function. The upper panel represents the transcriptional overlap of *dnmt1* 72h (red), *dnmt3* 72h (green) and *dnmt4* 72h (blue). Of all the genes analyzed only the two-fold up-regulated genes were plotted in this chart. This profile suggests that *dnmt1*, *dnmt3*, and *dnmt4* may have both common and discrete target genes. However, the genetic targets that the different DNA methyltransferases silence in a tissue-specific manner are not known. The lower panel represents a schematic of promoter methylation by DNA methyltransferases 4 silencing a gene. However, the genes that are methylated by DNA methyltransferases, and proteins that recruit DNA methyltransferase 4 to its target genetic loci warrant further elucidation. It is hypothesized that *dnmt4* may regulate the expression of hematopoietic genes that may regulate terminal differentiation of erythrocytes and neutrophils by this mechanisms

5.6 References

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APPENDIX A

CHARACTERIZATION OF MBD PROTEINS

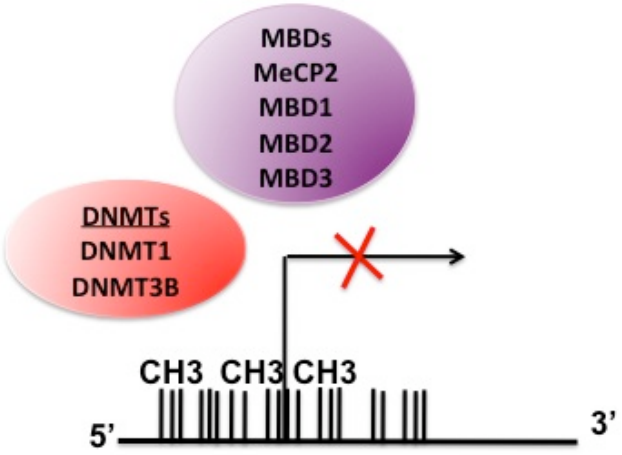

Figure A.1. Methylated DNA binding proteins and DNMTs. Methylated DNA binding proteins (MBDs) are considered as readers of the DNA methylation mark. The MBD proteins have a methyl-binding motif-MBD domain, a transcriptional repression domain-TRD and a cysteine rich domain –CxxC motif. All of the MBD proteins apart from MBD3 can bind methylated DNA. These proteins bind methylated DNA with the help of MBD motifs with the exception of Kaiso a member of the MBD proteins that does not harbor an MBD motif.

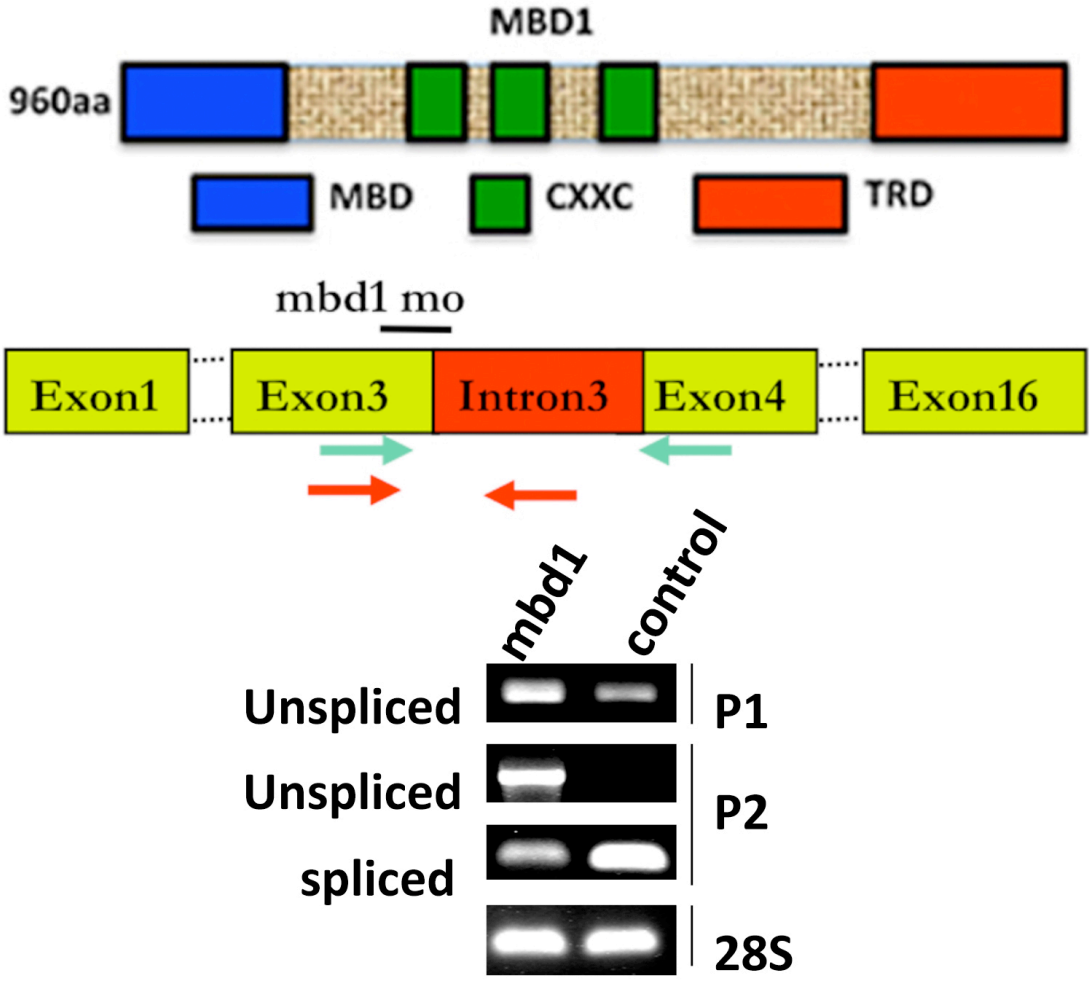

Figure A.2. Characterization of *mbd1* knockdown in zebrafish embryos. The upper panel shows the domain structure of the *mbd1* protein in zebrafish. The middle panel represents the design of the morpholino and the primers used for confirming splice check in the *mbd1* morphants at 24h. The lower panel shows a reduction of spliced product in the morphant embryos in comparison to the control embryos.

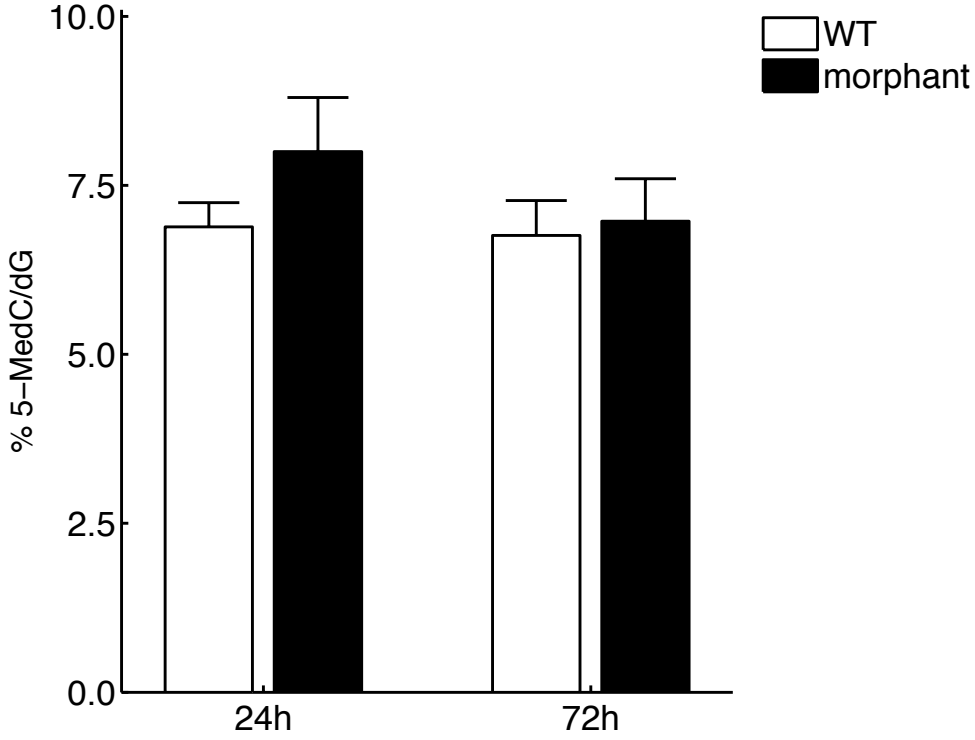

Figure A.3. Bulk methylation levels in *mbd1* morphants. The Y-axis in the graph represents the % 5mC levels (bulk cytosine methylation level) in *mbd1* and wild type control embryos at 24h and 72h. There is no apparent change in the bulk methylation levels on knocking down *mdd1* suggesting that *mbd1* is not involved in maintaining the bulk methylation levels in zebrafish embryos. However, it will be interesting to determine if there are subtle changes in the methylation levels of individual genes in *mbd1* morphants.

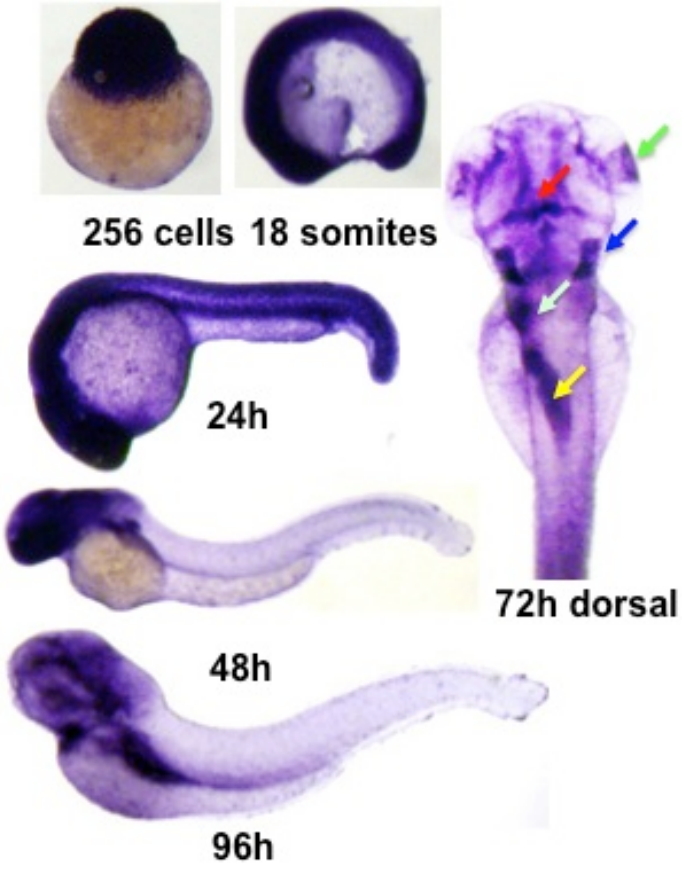

Figure A.4. Expression pattern of mbd1 in zebrafish. The transcripts of *mbd1* are maternally supplied. *mbd1* is ubiquitously expressed in earlier time points. At around 48h the expression is more robust in the eyes and the head. At 72h the expression of *mbd1* is restricted to the gut (yellow arrow), pancreas (white arrow), thymi (blue arrow), tegmentum (red arrow) and eyes (green arrow). The expression pattern of *mbd1* morphants suggests that it may be involved in molecular functions in particular tissues.

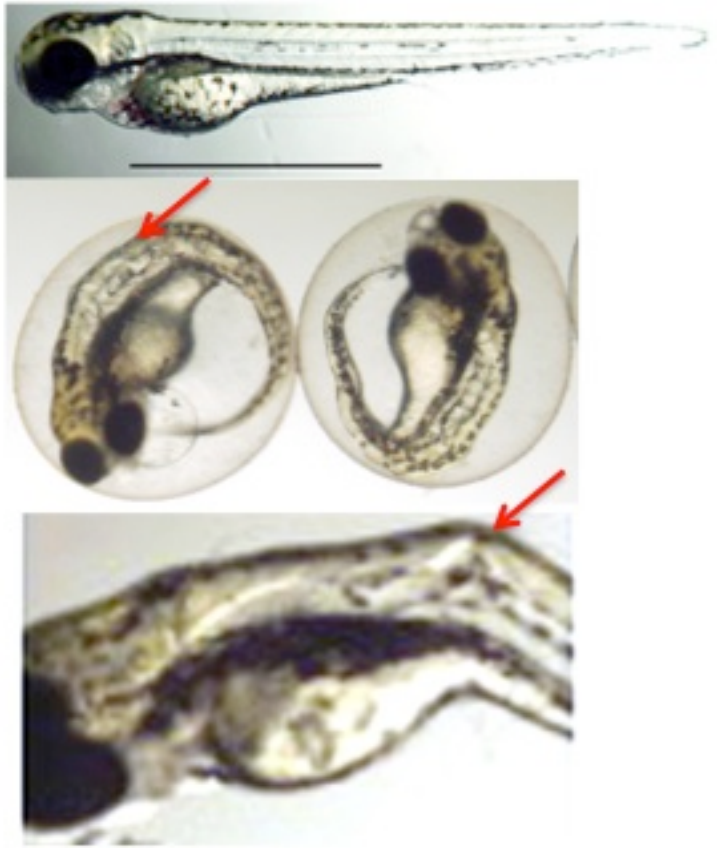

Figure A.5. The gross morphological phenotype of *mbd1* morphant embryos at 72h. On knocking down the levels of *mbd1* zebrafish embryos an interesting phenotype was observed. The 70% of embryos exhibited a cork screw shape phenotye at 48h and 72h. A similar phenotype is observed in *atp7a* mutant calamity. *Atp7a* is a gene that posses a CXXC motif like *mbd1*. It will be interesting to determine if *mbd1* functions like *atp7a* in regulating molecular functions.

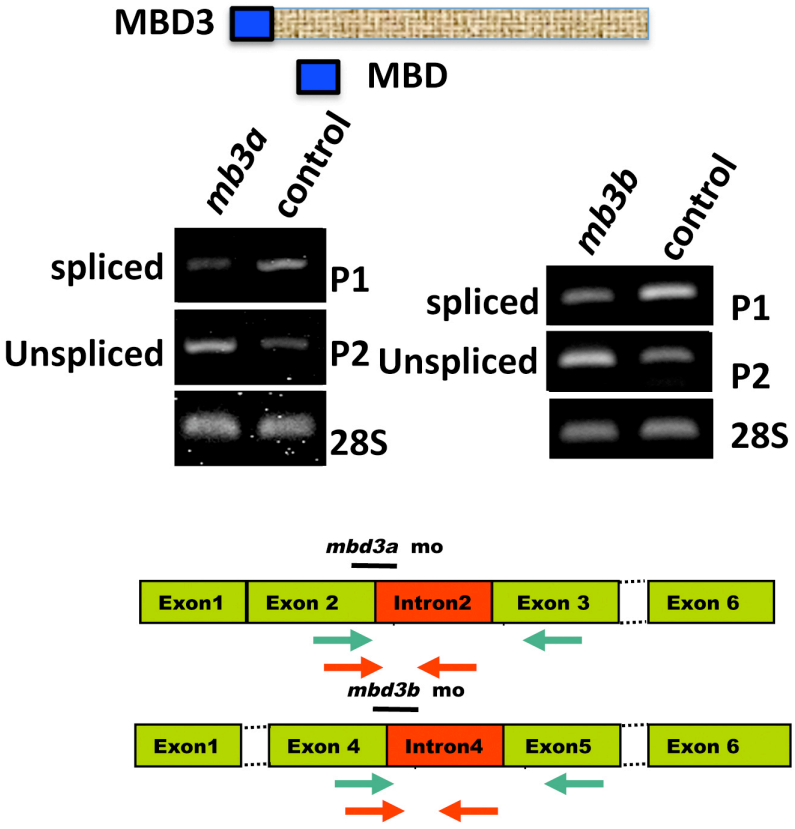

Figure A.6. Characterization of *mbd3a* and *mbd3b* knockdown in zebrafish. The upper panel shows the domain structure of the *mbd3* proteins in zebrafish. The middle panel shows the reduction in formation of spliced product in the morphant embryos in comparison to the control embryos. The lower panel represents the design of the morpholino and the primers used for confirming splice check in the *mbd3a* and *mbd3b* morphants at 24h.

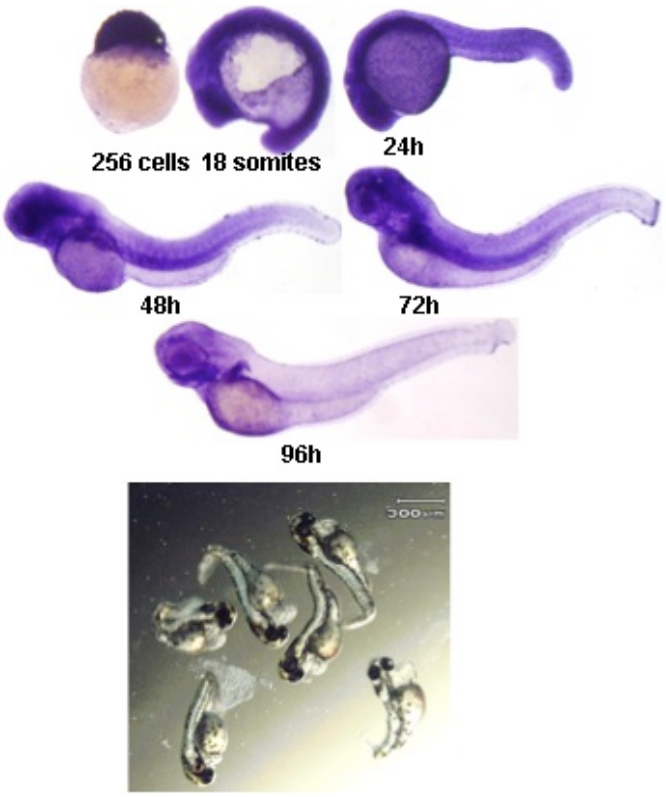

Figure A.7. Expression pattern of *mbd3a* and phenotypes of *mbd3a* morphants. The transcripts of *mbd3a* are maternally supplied and are ubiquitously expressed at all time points during development suggesting that it might have important functions in embryonic development. The upper panel shows the gross morphological defects of *mbd3a* morphants at 72h. The morphant displays a smaller head, closer set eyes (suggesting jaw defects), pericardial edema, and tail curvature defect.

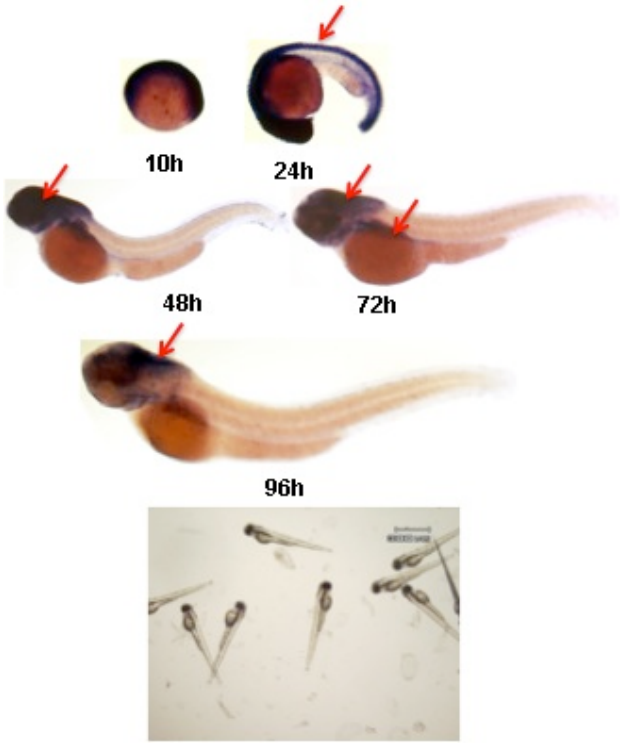

Figure A.8. Expression pattern of *mbd3b* and phenotypes of *mbd3b* morphants. The upper panel represents the expression pattern of *mbd3b.* The transcripts of *mbd3b* are maternally supplied and are ubiquitously expressed at early time points like 10h. At 24h the expression is robust in the neural tissues and the notochord. At 48h, 72h and 96h the expression is very restricted to the anterior structures like the eyes, brain pharyngeal arches and the pectoral fin. Interestingly, the knockdown of *mbd3b* does not show an apparent phenotype in the 72h morphants suggesting that the morphants may have subtle developmental defects.

APPENDIX B

COMPARISON OF OVEREXPRSSION AND KNOCKDOWN OF DNMT4

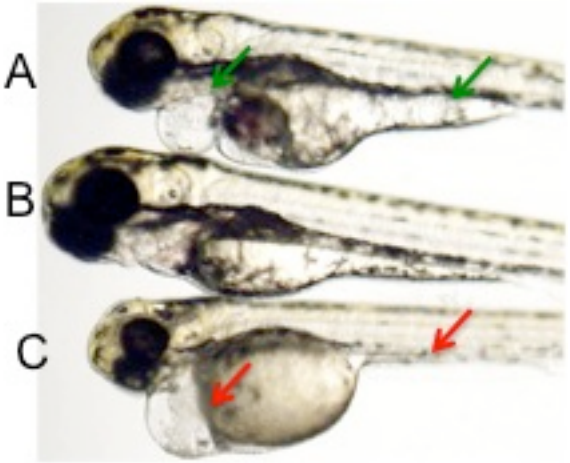

Figure B.1. Comparison of over-expression and knock-down of *dnmt4* . The upper panel (A) shows over expression of 0.15 pg of *dnmt4* RNA in wild type embryos. The embryos at 72h show abnormal yolk sac (green arrow) in comparison to uninjected control embryo in (B). Also the heart appears more looped than the uninjected embryo heart in (A). The embryos display strong pericardial edema and accumulation of RBC. In the lower panel (C) the *dnmt4* morphants display pericardial edema and hypochromic blood cell. The looping of the heart appears to be disrupted (red arrow). Also, the morphant embryos display lack of yolk sac extension.